IKKα regulation of canonical NF-κB activation downstream of Nod1-mediated peptidoglycan recognition

and

Endocytosis-independent function of clathrin heavy chain in the control of basal NF-κB activation

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ABSTRACT

NF-κB is a transcription factor involved in the regulation of inflammation and innate immunity. The IκB kinase (IKK) complex contains two catalytic subunits, IKKα and IKKβ, and plays an essential role in the activation of NF-κB through the phosphorylation and degradation of the NF-κB inhibitor IκBα, thereby allowing translocation of NF-κB into the nucleus. Numerous evidences indicate that IKKβ mediates NF-κB activation in response to pro-inflammatory cytokines and microbial products, but the role of IKKα in inflammation and innate immunity is unknown.

In the first part of dissertation, we focus on understanding the previously unknown function of IKKα in the canonical NF-κB pathway, associated with inflammation and innate immunity. We show that silencing of IKKα by RNA interference (RNAi) significantly reduced phosphorylation and degradation of IκBα, and nuclear translocation of NF-κB, and secretion of the pro-inflammatory chemokine interleukin-8 (IL-8) during *Shigella flexneri* infection of human epithelial HeLa cells. This suggests that IKKα like IKKβ plays a pivotal role in inflammation and innate immunity by mediating NF-κB activation in response to microbial infection.

Proper control of NF-κB activation is essential for inflammation and innate immunity triggered by microbial infection, but the dysregulation of NF-κB is associated with various diseases such as chronic inflammatory diseases and cancers. Thus, the NF-κB pathway has been a target of therapeutic drug development. Although constitutive and excessive NF-κB activation has been detected in many inflammation-related diseases, the cause of the constitutive NF-κB activation in non-stimulated cells is largely unknown.

In the second part of dissertation, we focus on clathrin heavy chain (CHC), a well-known regulator of endocytosis that plays a novel endocytosis-independent function as an inhibitor of basal NF-κB activation. We show that silencing of CHC induced constitutive NF-κB nuclear translocation and high level of IL-8 secretion in resting cells. We revealed that constitutive NF-κB nuclear translocation was mediated through the constant IκBα degradation in an IKKα-dependent mechanism. We further showed that CHC depletion-induced constitutive IκBα degradation and high level of IL-8 secretion in resting cells was independent of the inhibition of clathrin-mediated endocytosis (CME) as silencing of μ2 subunit of AP2 complex (AP2M1), an adaptor protein essential for CME failed to induce the constitutive IκBα degradation and high level of IL-8 secretion. Therefore, the results presented may suggest a potential link between a defect in CHC expression and chronic inflammatory disorders and cancers.

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LIST OF ABBREVIATIONS

ABIN	A20 binding inhibitor of NF-κB
ANK	ankyrin-repeat
AP2M1	μ2 subunit of AP2 complex
ATF	cyclic AMP-dependent transcription factor
BCL-3	B-cell lymphoma 3
BIR	baculovirus inhibitor of apoptosis repeat
CARD	caspase recruitment domain
CC	coiled-coil
cDNA	complementary DNA
CHC	clathrin heavy chain
CHUK	conserved helix-loop-helix ubiquitous kinase
CKII	casein kinase II
CLC	clathrin light chain
CLR	C-type lectin receptor
CME	clathrin-mediated endocytosis
C-terminal	carboxyterminal
CYLD	cylindromatosis tumor suppressor protein
DAP	diaminopimelic acid
DD	death domain
DNA	dioxyribonucleic acid
Dub	deubiquitination enzyme
ELISA	enzyme-linked immunosorbent assay
GPCR	G-protein-coupled receptor
GRR	glycin-rich region
H.pylori	*Helicobacter pylori*
HAT	histone actetyltransferase
HDAC	histone deacetylase
HLH	helix-loop-helix
HRP	horseradish peroxidase
HSP	heat shock protein
iE-DAP	D-g-Glu-meso-diaminopimelic acid
IKK	inhibitor of NF-κB (IκB) kinase
IL	interleukin
IL-1R	IL-1 receptor
IRAK	interleukin-1 receptor-associated kinase
IκB	inhibitor of NF-κB
JNK	c-Jun N-terminal protein kinase
K48	lysine 48
K63	lysine 63
L.monocytogenes	*Listeria monocytogenes*
LLO	listeriolysin O
LPS	lipopolysaccharide

LT	lympotoxin
LZ	leucine zipper
MAP3K	mitogen-activated protein kinase kinase kinase
MAPK	mitogen-activated protein kinase
MDP	muramyl dipeptide
MMP	matrix metalloproteinase
MOI	multiplicity of infection
mRNA	messenger RNA
MSK1	mitogen- and stress-activated protein kinase1
MyD88	myeloid differentiation primary response gene 88
NAG	N-acetylglucosamine
NAM	N-acetyl muramic acid
NBD	NEMO binding domain
NEMO	NF-κB essential modulator
NF-κB	nuclear factor kappa-B
NLR	NOD-like receptor
NLS	nuclear localization signal
NOD	nucleotide binding oligomerization domain
NRR	leucine-rich repeat
N-terminal	amionterminal
OspB	outer Shigella protein B
OspF	outer Shigella protein F
OspG	outer Shigella protein G
OUT	ovarian tumor
PAMP	pathogen-associated molecular patterns
PBS	phosphoate buffered saline
PCR	polymerase chain reaction
PEST	proline-, glutamic acid-, serine- and threonine-rich
PFA	paraformaldehyde
PGN	peptidoglycan
PI3K	phosphoinositide-3-kinase
PKA	protein kinase A
PKB	protein kinase B
PMN	polymorphonuclear leukocyte
PP	protein phosphatase
PRR	pattern recognition receptor
RANK	receptor activator of NF-κB
Rb	retinoblastoma protein
Rel	reticuloendotheliosis oncogene
RHD	Rel homology domain
RIG	retinoic acid-inducible gene
RIP	receptor interacting protein
RLR	RIG-I-like receptor
RNA	ribonucleic acid
RNAi	RNA interference

S.flexneri	*Shigella flexneri*
SCF	Skp1-Cdc53/Cullin1-F-box
siRNA	small interfering RNA
SODD	silencer of death domain
T3SS	type III secretion system
TAB	TAK1 binding protein
TAD	transactivation domain
TAK	TGF-beta activated kinase
TANK	TRAF family member-associated NF-κB activator
TBK1	TANK-binding kinase 1
TD	transactivation domain
TfR	transferrin receptor
TGF	transforming growth factor
TLR	toll-like receptor
TNF	tumor necrosis factor
TNFR	TNF receptor
Tollip	Toll-interacting protein
Tom1	target of Myb1
TRAF	TNF receptor-associated factor
TrCP	transducin repeat-containing protein
Tri-DAP	L-Ala-D-γ-Glu-meso-diaminopimelic acid
TSB	tryptic soy broth
UBC	ubiquitin-conjugating enzyme
UV	ultraviolet
ZF	zinc finger

CHAPTER 1

GENERAL INTRODUCTION

1.1 The transcription factor NF-κB

1.1.1 NF-κκB and IκB proteins

Nuclear factor kappa-B (NF-κB) was originally discovered by Sen and Baltimore in 1986 as a transcription factor present in activated B-cells that strongly activates the immunoglobulin κB light chain gene expression (Sen and Baltimore 1986). 25 years of research on NF-κB has revealed that this transcription factor plays important roles in diverse physiological responses including inflammation, adaptive immunity, cell adhesion, cell growth, differentiation, oxidative stress responses and apoptosis (Gilmore 2006). Not surprisingly, dysregulation of NF-κB has been implicated in an ever-expanding list of diseases such as Crohn's disease, arthritis, diabetes and cancers (Lawrence 2009).

There are five known members of the mammalian NF-κB/Rel family forming various homo- and hetero-dimers: RelA (p65), RelB, c-Rel, NF-κB1 (p105/p50), and NF-κB2 (p100/p52) (Figure 1.1) (Ghosh and Hayden 2008). All the NF-κB proteins share a highly conserved Rel-homology domain (RHD) for dimerization, nuclear localization, and DNA binding. The Rel proteins (RelA, RelB and c-Rel) contain C-terminal transactivation domains. NF-κB1 (p105/p50) and NF-κB2 (p100/p52) are distinguished by their long C-terminal domains that contain multiple copies of ankyrin repeats, which act to inhibit these proteins. NF-κB1 (p105/p50) and NF-κB2 (p100/p52) become shorter and active DNA-binding proteins (p105 to p50 and p100 to p52) by limited proteolysis (Fan and Maniatis 1991; Betts and Nabel 1996). The processing of p105 and p100 is mediated by the

ubiquitin-proteasome pathway and involves selective degradation of their C-terminal region containing ankyrin repeats (Fan and Maniatis 1991; Betts and Nabel 1996). Glycine-rich region (GRR) provides the stop signal for processing of p105 and p100 (Lin and Ghosh 1996; Heusch, Lin et al. 1999). The most abundant form of NF-κB is the heterodimer of RelA (p65) and p50, retained in the cytoplasm through interaction with IκB proteins, which masks nuclear localization signal (NLS) of NF-κB proteins (Jacobs and Harrison 1998).

The IκB (NF-κB inhibitor) proteins include IκBα, IκBβ, IκBε, IκBζ, Bcl-3, IκBNS (NF-κBδ), and the NF-κB precursors p100 and p105 (Figure 1.1) (Ghosh and Hayden 2008). All IκBs contain five to seven ankyrin-repeats mediating the binding to the RHD masking the nuclear localization signal (NLS) of NF-κB. The best-characterized IκB proteins is IκBα, composed of three regions: an N-terminal region, which regulates signal-dependent degradation; an ankyrin repeat domain; and a C-terminal PEST region regulating basal degradation. Nearly all of the NF-κB is bound to IκBα, resulting in near-complete inhibition of nuclear localization and transcriptional activation (Ferreiro and Komives).

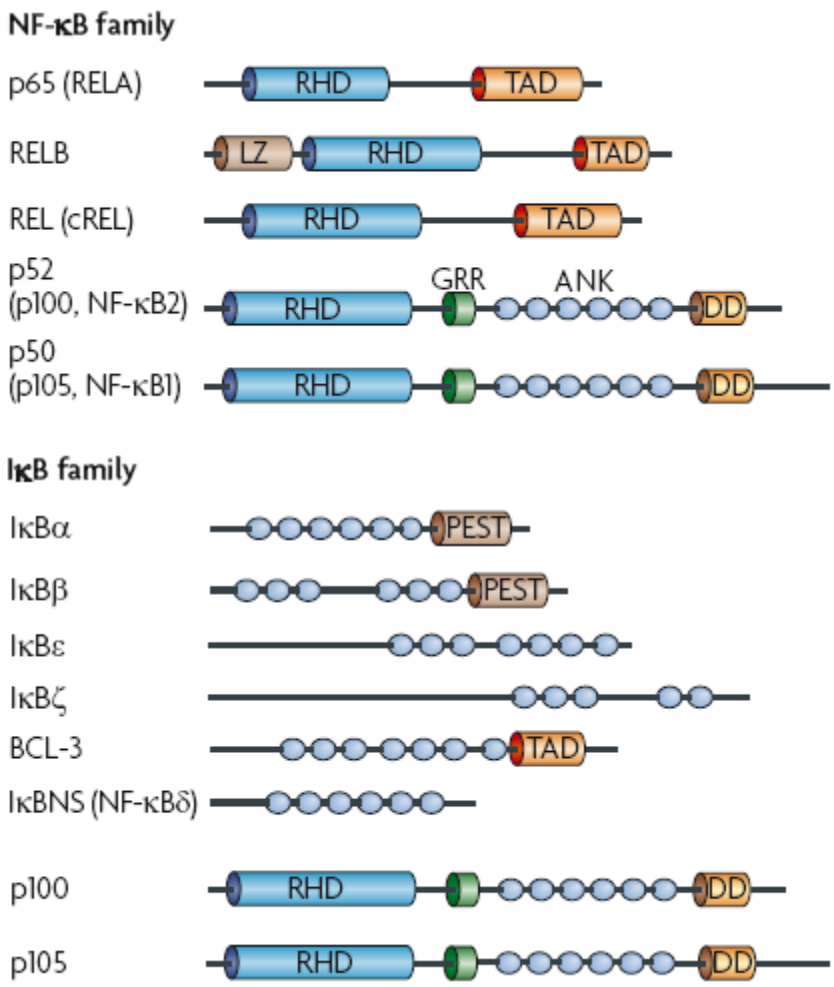

Figure 1.1. NF-κB and IκB protein families. Domains for each protein family are shown. The precursor proteins p100 and p105 function as both IκB proteins and, when processed by the proteasome, NF-κB family members. ANK, ankyrin-repeat; BCL-3, B-cell lymphoma 3; DD, death domain; GRR, glycine-rich region; PEST, proline-, glutamic acid-, serine- and threonine-rich; RHD, REL homology domain; TAD, transactivation domain. Adapted from Ghosh and Hayden 2008 (Ghosh and Hayden 2008)

1.1.2 Canonical and non-canonical NF-κB signaling pathways

With the exception of mature B cells where they are constitutively nuclear, in all other cell types NF-κB dimers are present in the cytoplasm through association with the IκBs as inactive forms (Liou and Baltimore 1993). Activation of NF-κB (usually assessed by the presence of nuclear NF-κB) is induced by diverse extracellular stimuli including inflammatory cytokines such as tumor necrosis factor alpha (TNFα) and interleukin-1 (IL-1), receptor ligands such as CD40-ligand, physical stress such as ultraviolet (UV) irradiation, and many bacteria and viruses (Pahl 1999). The active NF-κB promotes the expression of hundreds of target genes including cytokines, chemokines, cell adhesion molecules, stress response genes, and the regulators of apoptosis [nf-kb.org]. A key step for controlling NF-κB activity is the regulation of the NF-κB- IκBα interaction.

There are at least two separate pathways for NF-κB activation; the canonical and noncanonical pathway (Gilmore 2006; Lawrence 2009). The canonical (or classical) pathway is triggered by proinflammatory cytokines such as TNFα and IL-1, and microbial products such as lipopolysaccharide (LPS) and peptidoglycan (PGN), and is associated with inflammation. In the canonical pathway, NF-κB (for example, the RelA (p65)/p50 complex) activation is mediated by IKK complex, especially IKKβ and NEMO/IKKγ, dependent phosphorylation and degradation of IκBs (Figure 1.2) (Karin and Ben-Neriah 2000). In contrast, the noncanonical pathway depends on IKKα-dependent processing of the p100 precusor into p52 and subsequent activation of RelB/p52 complex (Dejardin 2006; Lawrence and Bebien 2007). The noncanonical (or alternative) pathway is activated by TNF-family cytokines including lymphotoxinβ (LT or TNFSF3), CD40 ligand (CD40L or TNFSF5), B cell activating factor (BAFF or TNFSF13B), and receptor activator of NF-

κB ligand (RANKL or TNFSF11), and is important in lymphoid organogenesis and B-cell development (Gerondakis and Siebenlist).

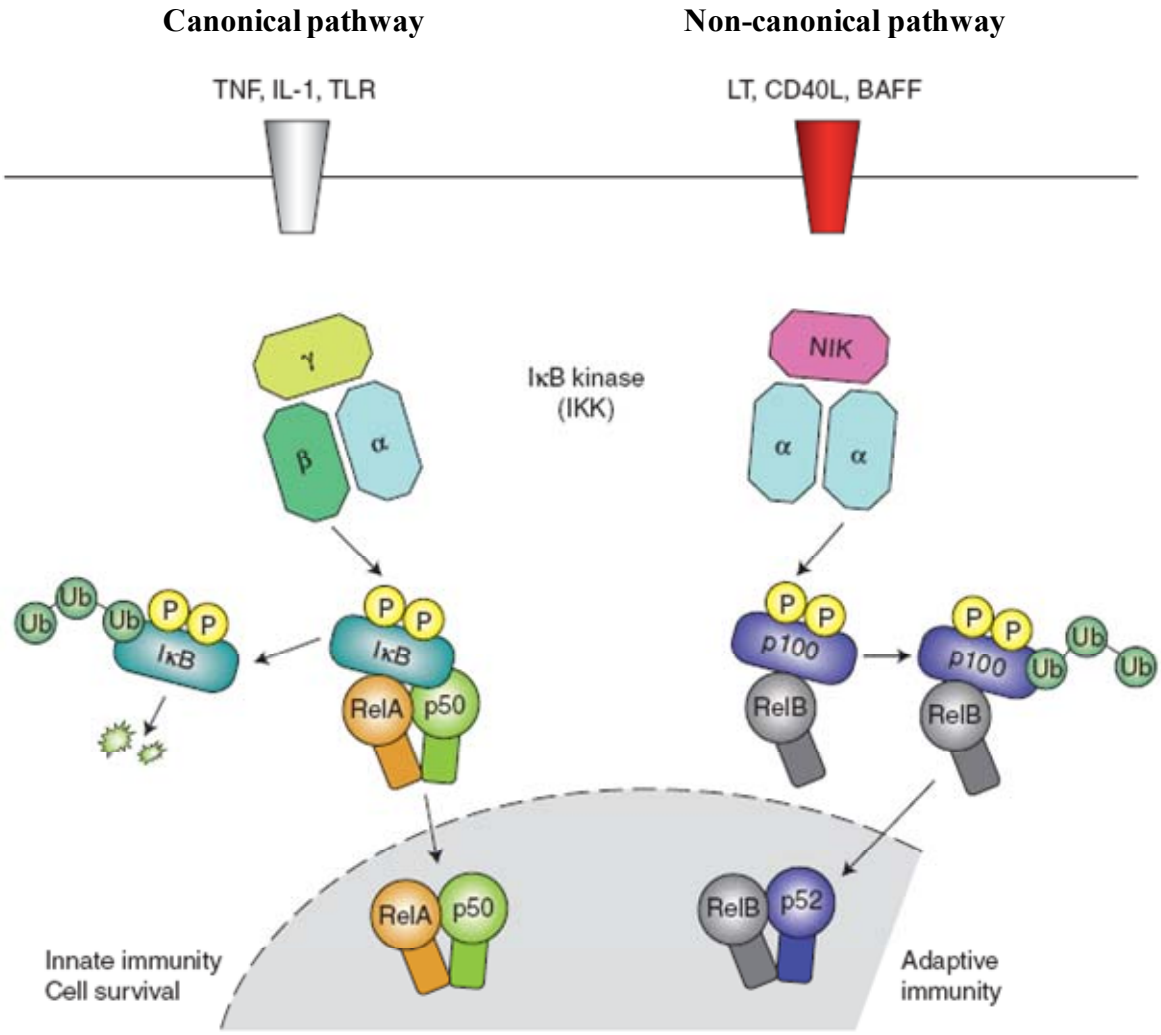

Figure 1.2 Canonical and noncanonical NF-κB pathways. The canonical pathway is triggered by TLRs and proinflammatory cytokines such as TNFα and IL-1, leading to activation of RelA that regulates expression of proinflammatory and cell survival genes. The noncanonical NF-κB pathway is activated by LTβ, CD40L, BAFF, and RANKL, but not TNFα, and results in activation of RelB/p52 complexes. Activation of the alternative pathway regulates genes required for lymph-organogenesis and B-cell activation. These pathways are characterized by the differential requirement for IKK subunits. IKKβ regulates activation of the canonical pathway through phosphorylation of IκBs and requires the IKKγ subunit but not IKKα, whereas IKKα is required

for activation of the non-canonical pathway through the phosphorylation and processing of p100, the precursor for p52, but this is independent of both IKKβ and IKKγ. Adapted from Lawrence T. Cold Spring Harb Perspect Biol 2009 (Lawrence 2009).

1.1.3 Mechanisms of IKK complex activation and inhibition

The IKK complex is composed of at least two highly homologous kinase subunits, IKKα /CHUK and IKKβ, and a regulatory subunit IKKγ/NEMO (NF-κB essential modulator) (Figure 1.3) (Hacker and Karin 2006). Based on mutational analyses, it is generally believed that IKKβ and NEMO are essential for IκBα phosphorylation and degradation in most canonical NF-κB signalling pathways, whereas IKKα is dispensable in the canonical pathway, but is essential for p100 phosphorylation and processing to p52 in the non-canonical pathway. In addition to the core IKK components IKKα, IKKβ, and NEMO, additional subunits are reported to associate with the IKK complex. HSP-90/Cdc37 functions as a chaperone during assembly of the IKK complex upon stimulation (Hinz, Broemer et al. 2007). The HSP-90 inhibitor geldanamycin has been shown to inhibit activation of IKK by TNFα (Lewis, Devin et al. 2000). ELKS is reported to be associated with IKK complex as a regulatory component like NEMO (Ducut Sigala, Bottero et al. 2004).

IKKα and IKKβ dimerize through the leucine zipper domain, which is also required for kinase activity (Mercurio, Zhu et al. 1997; Woronicz, Gao et al. 1997; Zandi, Rothwarf et al. 1997). IKKα and IKKβ bind NEMO through the C-terminal NEMO-binding domain (NBD) (Figure 1.4) (Hayden and Ghosh 2008). The N-terminal coiled-coil motif of NEMO is responsible for the interaction with IKKα and IKKβ (Drew 2007). Activation of IKK

complex requires NEMO oligomerization and serine 177 and serine 181 phosphorylation of IKKβ within the activation loop (T loop). IKKα is similarly phosphorylated on serine residues 176 and 180. An enforced NEMO oligomerization leads to IKK activation (Inohara, Koseki et al. 2000; Poyet, Srinivasula et al. 2000). Mutation of the activation loop serines to glutamic acid makes IKK constitutively active, while mutation to alanines abrogates downstream signal transduction (Hacker and Karin 2006). In the resting state, the IKK complex is held inactive through its interaction with NEMO. Upon stimulation, NEMO undergoes ubiquitination and oligomerization, which induces a conformational change that leads to the exposure of kinase domain and T loop serines on IKKα and IKKβ, thereby allowing transautophosphorylation or T-loop serine phosphorylation by upstream kinases such as TAK1 (Hayden and Ghosh 2008).

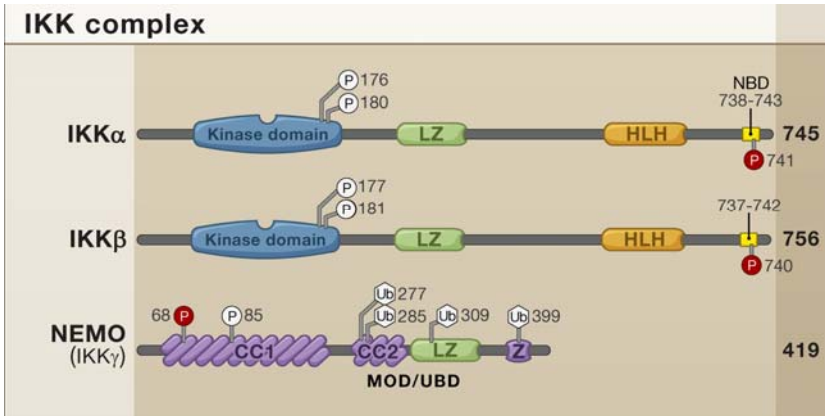

Figure 1.3 The IKK Protein Family. Members of the IKK proteins are shown. The number of amino acids in each human protein is indicated on the right. Posttranslational modifications that influence IKK activity or transcriptional activation are indicated with P or Ub for phosphorylation or ubiquitination, respectively. LZ, leucine zipper domain; HLH, helix-loop-helix domain; NBD, NEMO-binding domain; CC1/2, coiled-coil domains; Z, zinc finger domain. Adapted from Hayden and Ghosh 2008 (Hayden and Ghosh 2008).

Activation of IKK activity is a transient event, requiring negative feedback regulation. It was shown that phosphorylation of the NBD at serine 740 in IKK intrinsically inhibits IKK activity (May, D'Acquisto et al. 2000). In addition, active IKK can phosphorylate serine 68 within the IKK-binding domain of NEMO, which disrupts the interaction between IKK and NEMO, thereby terminating signalling (Palkowitsch, Leidner et al. 2008). The serine 68 phosphorylation in NEMO and serine 740 phosphorylation in IKKβ allows dephosphorylation of serines in the T-loops by PP2A or PP2Cβ, resulting in reconstitution of a signalling-competent IKK complex through Cdc37/HSP-90-mediated chaperone activity (Figure4) (Kray, Carter et al. 2005; Palkowitsch, Leidner et al. 2008).

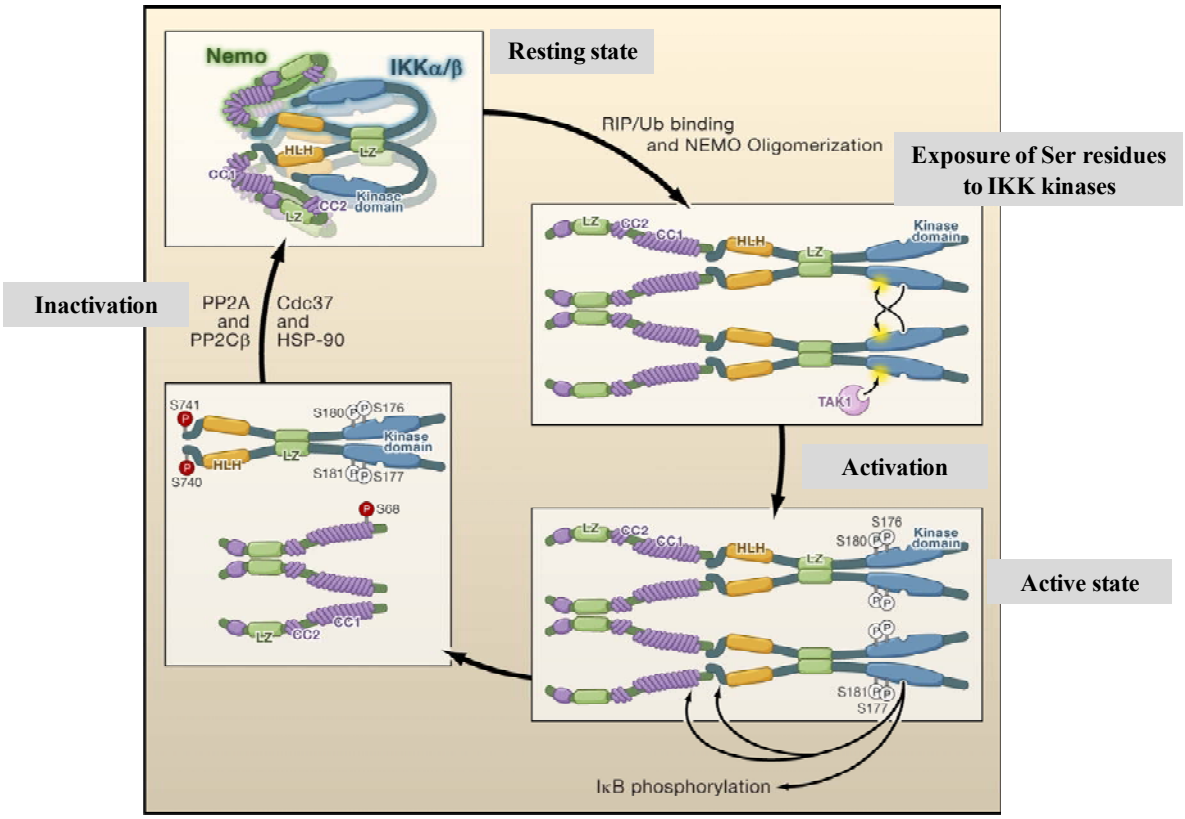

Figure 1.4 A putative model for IKK activation. In the resting state, activation of IKKα and IKKβ is prevented by binding to NEMO. Conformational changes in the IKK complex induced by binding of NEMO to RIP, and/or ubiquitination of NEMO, lead to the exposure of the IKK kinase domain

and T loop serines and consequent transautophosphorylation or phosphorylation by an IKK-K such as TAK1. The active IKK then phosphorylates downstream substrates, including serine 740 within the IKK NBD and serine 68 in NEMO. NEMO phosphorylation results in the separation of stable NEMO dimers and NEMO binding to IKK. Dephosphorylation of the IKK T loop results in kinase inactivation, whereas phosphorylation of the IKK NBD and NEMO serine 68 prevents reactivation of the kinase. Cdc37/ HSP-90-mediated chaperone activity and PP2A and PP2Cβ phosphatase activity may then mediate regeneration of the IKK complex. Adapted from Hayden and Ghosh 2008 (Hayden and Ghosh 2008).

1.1.4 Activation of the NF-κB pathway by ubiquitin signaling

Ubiquitination is a reversible covalent modification by which ubiquitin is attached to a target protein through an isopeptide bond between the C-terminus of ubiquitin and the ε-amino group of a lysine residue in the target protein (Pickart and Eddins 2004). This process is catalyzed by three enzymatic steps via an ubiquitin-activating enzyme (E1), an ubiquitin-conjugating enzyme (E2 or UBC), and an ubiquitin-protein ligase (E3). Ubiquitin contains seven lysine residues that can be attached to other ubiquitins to form a polyubiquitin chain (Pickart and Eddins 2004).

A polyubiquitin chain linked through lysine 48 (Lys-48) of ubiquitin targets a protein for degradation by the proteasome (Pickart and Eddins 2004). This ubiquitin-proteasome pathway is responsible for the degradation of the NF-κB inhibitor IκBα in the canonical NF-κB signaling pathway or the processing of p100 in the non-canonical pathway (Figure 1.5) (Alkalay, Yaron et al. 1995; Chen, Hagler et al. 1995; Skaug, Jiang et al. 2009). In response to cytokines such as TNFα, IκBα is phosphorylated by IKKα/β at two serine residues near the N-terminus (Ser-32 and Ser-36) (DiDonato, Mercurio et al.

1996; DiDonato, Hayakawa et al. 1997) and is then ubiqutinated by an E2 of the UBC4/5 family and the $SCF^{\beta\text{-}TrCP}$ E3 ligase complex (Skp1-Cdc53/Cullin1-F-box ligase containing the β transducin repeat-containing protein β-TrCP) (Spencer, Jiang et al. 1999; Winston, Strack et al. 1999). The polyubiquitinated IκBα is then selectively degraded by the 26S proteasome (Chen, Hagler et al. 1995).

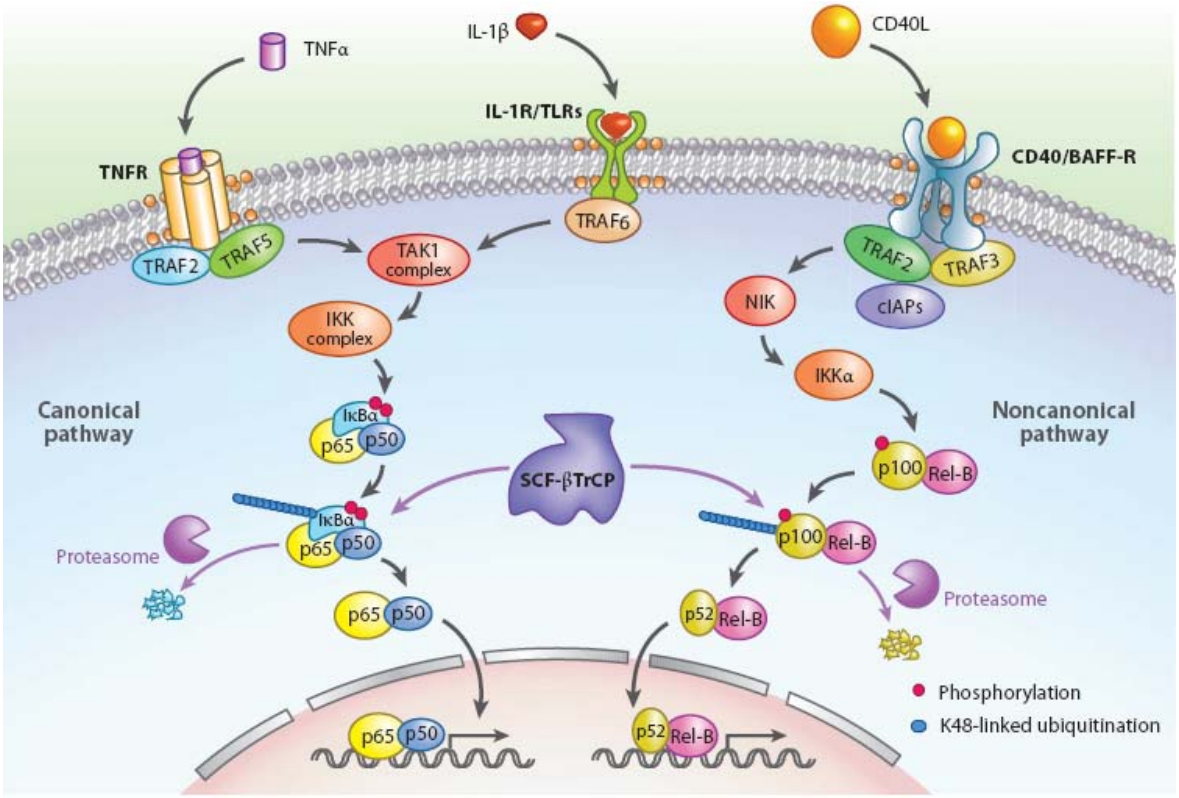

Figure 1.5 Degradation or processing of IκBα proteins by Lys48-linked ubiquitination. In canonical NF-κB activation (left), stimulation of the TNF receptor (TNFR), IL-1 receptor (IL-1R), and Toll-like receptors (TLRs) leads to activation of the TAK1 complex through TRAF proteins. TAK1 then activates IKK, which in turn phosphorylates IκB proteins and targets them for polyubiquitination by the SCF-βTrCP E3 ligase complex. Ubiquitinated IκB is degraded by the proteasome, allowing the p50/p65 NF-κB dimer to enter the nucleus and activate gene transcription. In noncanonical NF-κB activation (right), stimulation of a subset of receptors, including the BAFF receptor, leads to the stabilization of the kinase NIK, followed by activation of IKKα. IKKα phosphorylates p100, leading to its ubiquitination by the SCF-βTrCP complex. Ubiquitinated p100 is targeted for

proteasomal processing to p52. The p52/REL-B dimer then translocates into the nucleus to activate gene transcription.

On the other hand, a polyubiquitin chain linked through lysine 63 (Lys-63) of ubiquitin does not target a protein for degradation by the proteasome, but it plays a crucial role in the regulation (both activation and inhibition) of diverse NF-κB signaling pathways (Chiu, Zhao et al. 2009). The IKK complex integrates signals from diverse pathways to activate NF-κB (Solt and May 2008). TRAF (TNFR-associated factor) family of ubiquitin E3 ligases play an essential role in the activation of the IKK complex by many cell-surface receptors, including TNF receptor (TNFR) superfamily, the IL-1 receptor (IL-1R) and Toll-like receptors(TLRs) (Bradley and Pober 2001; Chung, Park et al. 2002). Binding of interleukin-1β (IL-1β) to IL-1R or LPS to TLR4 causes recruitment of MyD88, IRAK, and TRAF6 to the receptor. Auto-K63 polyubiquitination of TRAF6 facilitated by Ubc13/Uev1A (E2 ubiquitin conjugating enzyme) recruits and activates the TAK1 (TGFβ-activated kinase 1) complex and IKK complex through binding to the regulatory subunits, TAB2 and NEMO, respectively (Figure 1.6) (Deng, Wang et al. 2000; Wang, Deng et al. 2001; Kanayama, Seth et al. 2004; Skaug, Jiang et al. 2009). In the TNFR pathway, TRAF2 and TRAF5 (E3s) together with Ubc13 (E2) promote the Lys-63 polyubiquitination of RIP1 (receptor interacting protein 1) (Tada, Okazaki et al. 2001). The polyubiquitinated RIP1 then recruits and activates the TAK1 complex through the interaction with TAB2 or TAB3 (Kanayama, Seth et al. 2004). The polyubiquitin chains also recruit IKK complex by binding to NEMO, thus allowing TAK1 to phosphorylate and activate IKK (Wu, Conze et al. 2006).

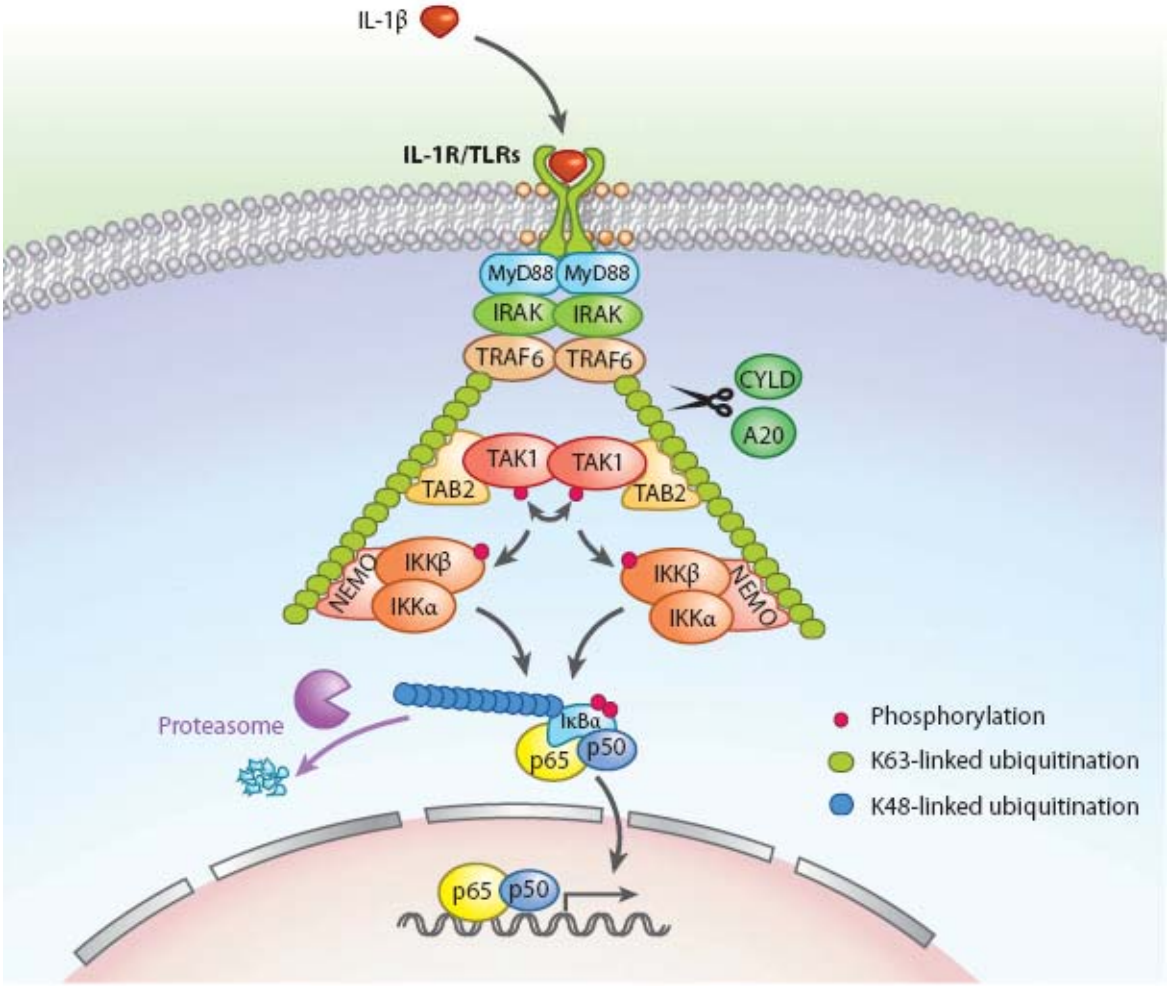

Figure 1.6 Regulation of NF-κB by Lys63-linked ubiquitination in the IL-1R/Toll-like receptor (TLR) pathway. Binding of interleukin-1β (IL-1β) to IL-1R or LPS to TLR4 causes recruitment of MyD88, IRAK, and TRAF6 to the receptor. TRAF6, an E3 ubiquitin ligase, catalyzes synthesis of Lys63-linked polyubiquitin chains, some of which are conjugated to TRAF6 itself. The polyubiquitin chains function as a scaffold to recruit the TAK1 and IKK complexes through binding to the regulatory subunits, TAB2 and NEMO, respectively. Recruitment of the kinase complexes facilitates autophosphorylation of TAK1 and subsequent phosphorylation of IKKβ by TAK1, leading to IκB degradation and subsequent activation of NF-κB (represented by the p50/p65 dimer). Lys63-linked polyubiquitin chains can be disassembled by deubiquitination enzymes including CYLD and A20, which inhibit IKK activation.

1.1.5 Inhibition of the NF-κB pathway by deubiquitination

Polyubiquitination is subject to disassembly by deubiquitination, which is carried out by members of deubiquitination enzymes (Dubs) (Amerik and Hochstrasser 2004). Two Dubs are best-characterized in inhibiting NF-κB activation to prevent uncontrolled NF-κB activities. CYLD (cylindromatosis tumor suppressor protein) inhibits IKK activation by cleaving K63-linked polyubiquitin chains on target proteins, including TRAF2, TRAF6 and NEMO following stimulation with TNFα or IL-1β (Figure 1.6) (Brummelkamp, Nijman et al. 2003; Kovalenko, Chable-Bessia et al. 2003; Trompouki, Hatzivassiliou et al. 2003; Skaug, Jiang et al. 2009). Overexpression of CYLD inhibits IKK and NF-κB activation, whereas RNAi of CYLD enhances IKK and NF-κB activation. However, little is known about how CYLD activity is regulated in resting cells and during stimulation.

A20 is an NF-κB induced Dub protein containing a ovarian tumor (OTU)-type Dub domain that inhibits NF-κB in a negative-feedback loop by cleaving K63-linked polyubiqutin chains on RIP and TRAF6 following stimulation with TNFα or IL-1β (Boone, Turer et al. 2004; Evans, Ovaa et al. 2004; Wertz, O'Rourke et al. 2004; Skaug, Jiang et al. 2009). Interestingly, A20 contains several zinc-finger domains through which it functions as an ubiquitin ligase to assemble K48-linked polyubiquitin chains on RIP after the K63 chains are cleaved by the OTU domain (Wertz, O'Rourke et al. 2004). K48 polyubiquitination targets RIP for degradation by the proteasome, further diminishing IKK activaton.

1.2 NF-κB signalling pathways during pathogen infection

1.2.1 Pathogen recognition by PRRs

The innate immune system is the first line of host defense against pathogens and recognizes microorganism such as bacteria via germline-encoded pattern recognition receptors (PRRs) (Takeuchi and Akira ; Medzhitov 2007). Different PRRs react with specific microbial components, known as pathogen-associated molecular patterns (PAMPs). Currently, four different classes of PRR families have been identified (Proell, Riedl et al. 2008). These families include transmembrane proteins such as the Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), as well as cytoplasmic proteins such as the Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and NOD-like receptors (NLRs) (see table 1).

TLRs are evolutionary conserved from *Caenorhabditis elegans* to mammals (Kawai and Akira). To date, 12 members of the TLR family have been identified in mammals. TLR2 in combination with TLR1 or TLR6 recognize lipoproteins (triacyl and diacyl lipoproteins, respectively), whereas TLR3, TLR7/TLR8, and TLR9 recognize nucleic acids (dsRNA, ssRNA and CpG-DNA, respectively). TLR4 and TLR5 recognize lipopolysaccharide (LPS) and flagellin, respectively (Akira, Uematsu et al. 2006). Recognition of bacterial components by TLRs takes place at either the cell surface or endolysosome compartments.

Table1. PRRs and their ligands. Adapted from Takeuchi and Akira, 2010 Cell (Takeuchi and Akira).

Table 1. PRRs and Their Ligands

PRRs	Localization	Ligand	Origin of the Ligand
TLR			
TLR1	Plasma membrane	Triacyl lipoprotein	Bacteria
TLR2	Plasma membrane	Lipoprotein	Bacteria, viruses, parasites, self
TLR3	Endolysosome	dsRNA	Virus
TLR4	Plasma membrane	LPS	Bacteria, viruses, self
TLR5	Plasma membrane	Flagellin	Bacteria
TLR6	Plasma membrane	Diacyl lipoprotein	Bacteria, viruses
TLR7 (human TLR8)	Endolysosome	ssRNA	Virus, bacteria, self
TLR9	Endolysosome	CpG-DNA	Virus, bacteria, protozoa, self
TLR10	Endolysosome	Unknown	Unknown
TLR11	Plasma membrane	Profilin-like molecule	Protozoa
RLR			
RIG-I	Cytoplasm	Short dsRNA, 5′triphosphate dsRNA	RNA viruses, DNA virus
MDA5	Cytoplasm	Long dsRNA	RNA viruses (Picornaviridae)
LGP2	Cytoplasm	Unknown	RNA viruses
NLR			
NOD1	Cytoplasm	iE-DAP	Bacteria
NOD2	Cytoplasm	MDP	Bacteria
CLR			
Dectin-1	Plasma membrane	β-Glucan	Fungi
Dectin-2	Plasma membrane	β-Glucan	Fungi
MINCLE	Plasma membrane	SAP130	Self, fungi

CLRs recognize carbohydrates from viruses, bacteria and fungi via a carbohydrate-binding domain (Geijtenbeek and Gringhuis 2009). For example, Dectin-1 and Dectin-2 are responsible for sensing β-glucans from fungi (Goodridge, Wolf et al. 2009; Robinson, Osorio et al. 2009). MINCLE, a CLR from macrophage, can sense not only fungal infection but also an endogenous protein, spliceosome-associated protein 130 (SAP130) from necrotic host cells (Yamasaki, Ishikawa et al. 2008).

RLRs are localized in the cytoplasm and primarily sense viral double stranded RNA (dsRNA) (Takeuchi and Akira 2009). RLRs are composed of two N-terminal caspase recruitment domains (CARDs), a central DEAD box helicase/ATPase domain, and a C-terminal regulatory domain that mediates the binding to dsRNAs. RIG-1 and MDA5 recognize relatively short (up to 1kb) and long (more than 2kb) double stranded RNA (dsRNA), respectively, and are essential for stimulating type I interferon (IFN) production in response to RNA viruses (Kato, Takeuchi et al. 2008; Loo, Fornek et al. 2008).

NLRs detect pathogens that have invaded the cytosol of host cells. The NLR family of proteins is defined by a tripartite structure consisting of a C-terminal leucine-rich repeat (LRR) that mediates ligand (pathogen) sensing; a central nucleotide binding oligomerization domain (NOD); and a N-terminal effector domain, such as CARDs, PYRIN, or baculovirus inhibitor of apoptosis repeat (BIR) domains (Inohara, Chamaillard et al. 2005; Martinon and Tschopp 2005) . In human, the NLR family is composed of 22 proteins (see the Table 2 for the list) (Proell, Riedl et al. 2008). Although primarily expressed in immune cells, including antigen-presenting cells such as macrophages and dendritic cells, NLRs can also be expressed in nonimmune cells, including epithelial cells (Chen, Shaw et al. 2009). Nod1 and Nod2 are the best-characterized NLRs and recognize

bacterial peptidoglycan (PGN) fragments D-γ-Glu-meso-diaminopimelic acid (iE-DAP) or L-Ala-D-γ-Glu-meso-diaminopimelic acid (Tri-DAP) and muramyl dipeptide (MDP), respectively (Figure 1.7) (Chamaillard, Hashimoto et al. 2003; Girardin, Boneca et al. 2003; Inohara and Nunez 2003).

Table 2. Overview of NLR family members according to their domain organization (Proell, Riedl et al. 2008).

Nomenclature	Synonyms	Domain structure	Chrom. location	Genebank
CARD domain				
NOD1	**NLRC1**,CARD4	CARD-NACHT-WH-SH-LRR	7p14.3	AF126484
NOD2	**NLRC2**, CARD15	CARD-CARD-NACHT-WH-SH-LRR	16q12.1	AF178930
CIITA type1	**NLRA**	CARD-(X-NACHT-WH-SH-LRR)	16p13.13	AF000002
PYRIN domain				
NALP1	NLRP1, CARD7	PYD-NACHT-WH-SH-LRR-FIIND-CARD	17p13.2	AB023143
NALP2	**NLRP2**, Pypaf2	PYD-NACHT-WH-SH-LRR	19q13.42	AK000517
NALP3	**NLRP3**, Pypaf1	PYD-NACHT-WH-SH-LRR	1q44	AF054176
NALP4	**NLRP4**, Pypaf4	PYD-NACHT-WH-SH-LRR	19q13.43	AF479747
NALP5	**NLRP5**, NOD14, Pypaf8	PYD-NACHT-WH-SH-LRR	19q13.43	AY154460
NALP6	**NLRP6**, Pypaf5	PYD-NACHT-WH-SH-LRR	11p15.5	AF479748
NALP7	**NLRP7**, NOD12, Pypaf3	PYD-NACHT-WH-SH-LRR	19q13.42	AF464765
NALP8	**NLRP8**, NOD16	PYD-NACHT-WH-SH-LRR	19q13.43	AY154463
NALP9	**NLRP9**, NOD6	PYD-NACHT-WH-SH-LRR	19q13.43	AY154464
NALP10	**NLRP10**, NOD8, Pynod	PYD-NACHT-WH-SH	11p15.4	AY154465
NALP11	**NLRP11**, NOD17, Pypaf6	PYD-NACHT-WH-SH-LRR	19q13.42	AY095145
NALP12	**NLRP12**, Pypaf7, RNO	PYD-NACHT-WH-SH-LRR	19q13.42	AY095146
NALP13	**NLRP13**, NOD14	PYD-NACHT-WH-SH-LRR	19q13.42	AY154468
NALP14	**NLRP14**	PYD-NACHT-WH-SH-LRR	11p15.4	BK001107
BIR domain				
NAIP	**NLRB1**, BIRC1	BIR-NACHT-WH-SH-LRR	5q13.1	U19251
untypical CARD				
Ipaf	**NLRC4**, Card12, Clan	Card?-NACHT-WH-SH-LRR	2p22-p21	AF376061
NOD4	**NLRC5**, NOD27	Card?-NACHT-WH-SH-LRR	16q13	AF389420
NOD3	**NLRC3**	Card?-NACHT-WH-SH-LRR	16p13.3	BK001112
undefined				
NOD5	**NLRX1**, NOD26, NOD9	X-NACHT-WH-SH-LRR	11q23.3	AB094095
CIITA	**NLRA**	X-NACHT-WH-SH-LRR	16p13.13	U18259

Protein name and synonyms (new synonyms in bold), accession number, and chromosomal location according to http://www.genenames.org/genefamily/nlr.php followed by domains as defined by FFAS. CARD, caspase activation and recruitment domain; PYD, pyrin; NACHT, domain present in NAIP, CIITA, HET-E, TP-1; NALP, NACHT-LRR-PYD-containing protein; WH, winged helix domain; SH, superhelical domain, LRR, leucine-rich repeats.
doi:10.1371/journal.pone.0002119.t001

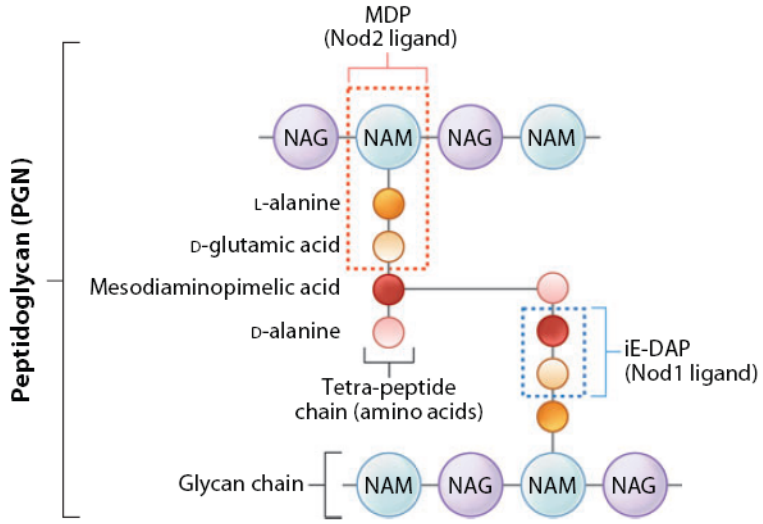

Figure 1.7 Nod1 and Nod2 ligands from bacterial peptidoglycan (PGN). Parallel PGN strands composed of the alternating amino sugars N-acetylglucosamine (NAG) and N-acetyl muramic acid (NAM) are crosslinked to each other by stem peptides. Cross-linking occurs via a direct link between a meso-diaminopimelic acid (meso-DAP) residue and the D-alanine residue in position four from a peptide anchored on the parallel glycan strand. Minimal motifs required for NOD1 and NOD2 (dashed boxes) recognition are also shown. Adapted from Chen et al. (Chen, Shaw et al. 2009).

1.2.2 NF-κB signal transduction during *Shigella* infection

Shigella flexneri (*S. flexneri*) was the first bacterium shown to be detected by the cytoplasmic bacterial sensor NOD1 (Girardin, Tournebize et al. 2001). The Gram-negative bacteria *Shigella* species including *S. flexneri, S. dysenteriae, S. boydii, and S. sonnei* invade the colonic and rectal epithelium of humans, causing an acute mucosal inflammation called Shigellosis that leads to the destruction of the colonic mucosa (Sansonetti 1998; Schroeder and Hilbi 2008).

S. flexneri invades intestinal epithelial cells by inducing cytoskeletal rearrangement localized at the site of infection (Bourdet-Sicard, Egile et al. 2000; Tran Van Nhieu, Bourdet-Sicard et al. 2000). This entry process depends on the activities of several effectors including IpaA, IpaB, IpaC and IpaD secreted from the *Shigella* type III secretion system (T3SS) (High, Mounier et al. 1992; Menard, Prevost et al. 1996; Tran Van Nhieu, Ben-Ze'ev et al. 1997; Tran Van Nhieu, Caron et al. 1999). The *Shigella* effector IpaC triggers actin polymerization and the formation of filopodial and lamellipodial extensions, which are dependent on the Rho small GTPases Cdc42 and Rac (Mounier, Laurent et al. 1999), and the protein tyrosine kinase Src (Dehio, Prevost et al. 1995). On the other hand, IpaA binds to the focal adhesion protein vinculin and induces depolymerization of actin filaments, allowing the transformation of the IpaC-induced extensions into a structure that is productive for bacterial entry (Tran Van Nhieu, Ben-Ze'ev et al. 1997).

Upon entry of *Shigella* into host cell, a peptidoglycan (PGN)-derived small peptide iE-DAP (γ-D-glutamyl-meso-diaminopimelic acid) is released into the cytosol by carboxy-peptidases and hydrolases activity (Boneca 2005), and sensed by NOD1 (Chamaillard, Hashimoto et al. 2003). Recognition of iE-DAP through leucine-rich repeat (LRR) domain induces self-oligomerization of NOD1 (Inohara, Koseki et al. 2000). Oligomerization of NOD1 allows binding to a downstream effector molecule RICK/RIP2 through CARD-CARD interaction (Park, Kim et al. 2007). RICK/RIP2 is then conjugated with lysine-63-linked polyubiquitin chains at lysine 209 (K209) located in its kinase domain by an unknown E3 ubiquitin ligase (Hasegawa, Fujimoto et al. 2008). Unlike K48-linked polyubiquitin chains, which target a protein for proteasomal degradation, the nondegradative K63-linked polyubiquitinated chains further recruit the TAK1 complex via

TAB2 or TAB3 (Chiu, Zhao et al. 2009). In addition, IKK complex is recruited to RICK/RIP2 through the interaction between NEMO and the intermediate domain (IM) of RICK/RIP2 (Hasegawa, Fujimoto et al. 2008). Thus, it was suggested that the proximity of TAK1 and IKK complex through the interaction with RICK/RIP2 may play an important role for NF-κB activation during Nod1 activation (Figure 1.8) (Hasegawa, Fujimoto et al. 2008). Activation of IKK complex by unknown mechanism leads to the phosphorylation of IκBα, which target IκBα for ubiquitination and subsequent degradation by the 26S proteasome (Hasegawa, Fujimoto et al. 2008). NF-κB is then free to translocate to the nucleus, where it induces expression of a variety of target genes encoding proinflammatory cytokines and chemokines, such as interleukin-8 (IL-8) (Chamaillard, Hashimoto et al. 2003; Arbibe, Kim et al. 2007). The deubiquitinase A20 is also induced by NF-κB, and is known to remove K63-linked polyubiquitin chains on RIP and TRAF6 (Boone 2004, Evans 2004). Thus, it is likely that A20 inhibits NF-κB in a negative-feedback loop by cleaving K63-linked polyubiqutin chains on RICK/RIP2 in Nod signaling (Figure 1.8).

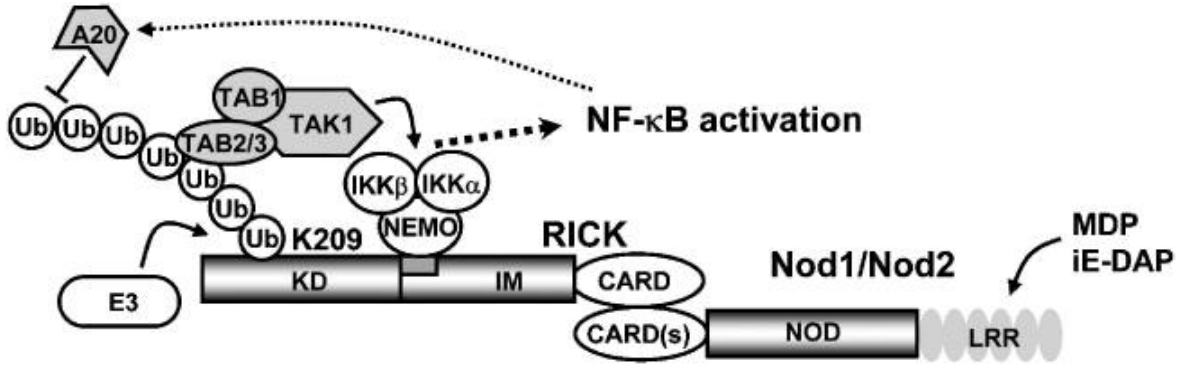

Figure 8. Model for NF-κB activation in Nod signaling. Adapted from Hasegawa et al.(Hasegawa, Fujimoto et al. 2008).

1.3 Function and transcriptional regulation of IL-8

1.3.1 IL-8-mediated inflammatory response to *Shigella* infection

When tissues get injured or infected by pathogens like *S.flexneri*, generally macro symptoms of redness, swelling, heat and pain appear. This process is called inflammation or inflammatory response. At the cellular level, inflammation is caused by chemical mediators called chemokines. Interleukin-8 (IL-8) is one of the major epithelial cells-secreted chemokines associated with inflammation (Jung, Eckmann et al. 1995). Secreted IL-8 then recruits phagocytes, in particular, neutrophils from the blood stream to the site of infection (Perdomo, Cavaillon et al. 1994; Kobayashi 2008). The function of IL-8 in inflammation during *S.flexneri* infection was shown in the rabbit model of shigellosis where intense IL-8 expression in the infected epithelial layer and neutrophil infiltration in the infected tissue was observed (Sansonetti, Tran Van Nhieu et al. 1999). Several recent studies have demonstrated that NF-κB activation is required but not sufficient to induce IL-8 expression upon *S.flexneri* infection (see below).

1.3.2 Transcriptional regulation of IL-8 by NF-κB

IL-8 promoter contains a NF-κB binding site known as kappa B (κB) element that is essential for transcriptional regulation of the gene (Mukaida, Mahe et al. 1990; Harant, de Martin et al. 1996). In line with the cytoplasmic retention of transcription factor NF-κB by its binding to IκB proteins, IL-8 expression is very low in unstimulated cells. Thus, nuclear translocation of NF-κB is critical for IL-8 production in response to a wide range of stimuli including proinflammatory cytokines such as TNFα, and bacterial products such as LPS or PGN (Philpott, Yamaoka et al. 2000). However, binding of the NF-κB element to the IL-8

gene promoter is not sufficient. The NF-κB components, especially p65 subunit needs to be phosphorylated in its transactivation domain (TD) to be fully active (Hoffmann, Natoli et al. 2006). Serine 276 phosphorylation in the TD of p65 by protein kinase A (PKA) (Zhong, Voll et al. 1998), casein kinase II (CKII) or protein kinase B (PKB or Akt) (Bird, Schooley et al. 1997), and serine 536 phosphorylation by IKKα and IKKβ (Sakurai, Chiba et al. 1999), PI3K/Akt (Sizemore, Leung et al. 1999) and IKKε/TBK1 (Buss, Dorrie et al. 2004; Adli and Baldwin 2006) are suggested to be required for its transactivation function. In addition to phosphorylation of NF-κB subunits, acetylation and methylation can modulate NF-κB transcriptional activity (Huang, Yang et al. ; Perkins 2006).

1.3.3 Epigenetic regulation of IL-8 gene

Epigenetic events such as histone acetylation and phosphorylation are known to play an important role in regulating gene expression (Munshi, Shafi et al. 2009). While repression of transcriptional activity is commonly correlated with histone hypoacteylation due to histone deacetylase (HDAC) activity, histone acetylation mediated by histone actetyltransferase (HAT) activity generally promotes transcriptional activation of genes after conformational changes within the chromatin (Kuo and Allis 1998; Wilson 2008). When it comes to epigenetic regulation of IL-8 gene, Wen et al. have demonstrated that the HDAC activity tightly controls the transcription of the IL-8 gene in Caco-2 intestinal epithelial cells (Wen and Wu 2001). Muegge et al. have also shown that histone H3 phosphorylation at serine 10 and acetylation at lysine 14 facilitates NF-κB binding to IL-8 promoter (Muegge 2002). Histone H3 phosphorylation at serine 10 is induced by mitogen- and stress-activated protein kinase 1 (MSK1) downstream of p38 or ERK signaling pathways (Thomson, Clayton et al. 1999). In addition, JNK contributes to IL-8 expression

through the activation of the transcriptional regulator AP-1 composed of c-JUN, ATF, c-FOS, and JDP families, which binds to AP-1-binding site present in the core IL-8 promoter (Hess, Angel et al. 2004; Bogoyevitch, Ngoei et al. 2010).

1.3.4 Subversion of host inflammatory signaling pathways by *Shigella* effectors

Subversion of host inflammatory signaling pathways is an important mechanism used by multiple bacteria (Bhavsar, Guttman et al. 2007). Especially, histone modifications induced by bacterial toxins are shared by multiple bacteria including *S.flexneri, Listeria monocytogenes and Helicobacter pylori (see below)*. For example, the *Shigella* type III effectors OspF (Outer *Shigella* protein F) induces dephosphorylation of p38 and ERK in the nucleus, which subsequently prevents histone H3 Ser10 phosphorylation (Arbibe, Kim et al. 2007; Li, Xu et al. 2007). *L.monocytogenes* secretes listeriolysin O (LLO), which induces a dramatic dephosphorylation of histone H3 at serine 10 (H3 Ser10) and deacetylation of histone H4 (Hamon, Batsche et al. 2007). Similarly, *H. pylori* induces cagPAI-dependent dephosphorylation of histone H3 at serine 10 and deacetylation of H3 lysine at lysine 23 (Ding, Fischer et al. 2010).

Besides OspF, *Shigella* use other effectors to downregulate the host inflammatory response. For example, OspB targets the nucleus to downregulate the host cytokine production via interactions with retinoblastoma protein (Rb) (Zurawski, Mumy et al. 2009). OspG binds to the ubiquitin-conjugating enzyme UbcH5b and inhibits the degradation of IκBα, blocking the NF-κB activation (Kim, Lenzen et al. 2005). IpaH9.8 is an E3 ubiquitin ligase that promotes the ubiquitin-binding adaptor protein ABIN-1 (A20 binding inhibitor of NF-κB)-dependent polyubiquitination and proteasome-dependent degradation of NEMO,

modulating the NF-κB activation and reducing NF-κB-mediated inflammatory response (Rohde, Breitkreutz et al. 2007; Ashida, Kim et al. 2010).

1.4 NF-κB and diseases

Given the fact that NF-κB controls hundreds of target genes involved in diverse cellular functions, it is not surprising that dysregulation of NF-κB has been implicated in an ever-expanding list of diseases such as immune deficiency, arthritis, diabetes and cancers (Karin and Greten 2005; Okamoto 2006). Epidemiological studies have shown that about 15% of human deaths from cancer are associated with chronic viral or bacterial infections (Karin and Greten 2005). It is thought that there are 1.2 million cases of infection-related maliganancies per year (Kuper, Adami et al. 2000; Bogoyevitch, Ngoei et al. 2010).

1.4.1 Mutations in NF-κB signaling pathways

Mutations in NF-κB signaling pathways have been associated with human diseases such as chronic inflammatory diseases and immune diseases by affecting expression of target genes (Courtois and Gilmore 2006). For example, multiple variants of mutations in Nod2 are closely linked to Crohn's disease, an inflammatory bowel disease, causing inflammation of intestine (Hugot, Chamaillard et al. 2001; Bonen, Ogura et al. 2003), whereas certain mutations in Nod1 are associated with an increased risk of developing asthma (Hysi, Kabesch et al. 2005). A point mutation at serine 32 residue of IκBα is associated with an impaired innate immune response and a severe immune deficiency as IκBα phosphorylation and degradation (and subsequent NF-κB activation) is impaired in cells with this mutation (Courtois, Smahi et al. 2003; Janssen, van Wengen et al. 2004).

1.4.2 NF-κB as a therapeutic target

Given the implication in many human diseases, the NF-κB pathway is a good therapeutic target. Over 785 inhibitors of the NF-κB pathway have been identified and the number is

keep growing (Gilmore 2006). Among those, a number of small chemical compound targeting IKK complex are under pre-clinical trials as a therapeutic intervention of cancers (Lee and Hung 2008). IKKβ specific inhibitors include PS-1145 (Hideshima, Chauhan et al. 2002), SPC-839 (Palanki 2002), ML120B (Wen, Nong et al. 2006) and SC-514A (Kishore, Sommers et al. 2003). One of the prominent features of cancer cells is resistance to apoptosis via NF-κB dependent anti-apoptotic gene expression. Thus, it is likely that NF-κB inhibitors can be used to sensitize cancer cells in response to apoptosis-inducing agents. A study with sodium salicylate and asprin known as NF-κB inhibitors has shown that these agents decreased NF-κB activation and high levels of the anti-apoptotic protein cFLIP expression in leukemic cells, allowing TNFα-induced apoptoiss (Kopp and Ghosh 1994).

1.5 Cellular functions of clathrin

No function of clathrin in NF-κB signalling pathways has been shown so far, but the second part of my thesis reveals a novel clathrin function in NF-κB signalling pathway. Thus, it is worth mentioning general information on clathrin in this section.

1.5.1 Clathrin-mediated endocytosis

Well-chracterized functions of clathrin include endocytosis of many receptors, channels, transporters as well as various soluble macromolecules and viruses ("cargo") (Conner and Schmid 2003). Several motifs for clathrin-dependent internalization are known including the tyrosine-based motif (YXXΦ), di-leucine-based motif, NPXY and mono-/multi-ubiquitination (Mousavi, Malerod et al. 2004). During internalization, adaptor proteins recognize trafficking motifs of cargo proteins, link them to clathrin, and concentrate them in clathrin-coated pits. The clathrin-coated pits invaginate into the cytoplasm, and eventually pinch off from the plasma membrane to form clathrin-coated vesicles in a GTPase dynamin-dependent manner (Figure 10) (Conner and Schmid 2003). The main component of clathrin-coated pits and vesicles is the clathrin triskelion, consisting of three heavy chain (CHC) and three light chain (CLC) (Kirchhausen 2000). Interactions between clathrin and adaptor proteins are mediated through the N-terminal domain of CHC and the clathrin boxes (LLpL[-] where L typically denotes a leucine, and p and [-] denote a polar and a negatively charged residue, respectively) in adaptor proteins. For example, AP-2 is a heterotetramer composed of α and β2 adaptins and μ2 and σ2 subunits. β2 adaptin contains the clathrin box LLNLD. Among others, the transferrin receptor (TfR) is well estabilished to be specifically internalized via the clathrin-dependent pathway, and therefore can be employed as a marker for clathrin-dependent endocytotic compartments. The μ2 subunit of

the AP-2 complex interacts with the internalization motif YXXΦ in the transferrin receptor (Owen, Collins et al. 2004). Although the light chain (CLC) is known to be required for efficient trimerization and heavy chain stability (Ybe, Greene et al. 1998), it is dispensable for the assembly of clathrin with AP complexes (Lindner and Ungewickell 1991) .

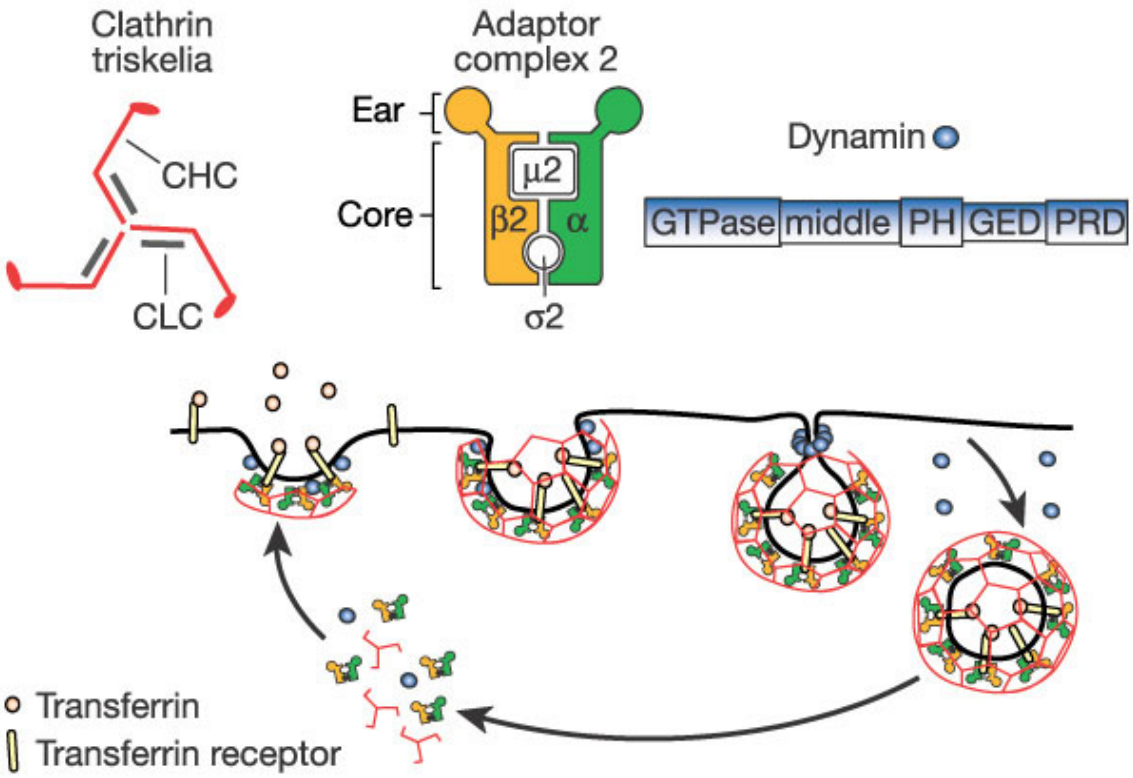

Figure 10. Clathrin triskelion, AP2 complex and dynamin-dependent endocytosis of transferrin-transferrin receptor. Clathrin triskelions, composed of three clathrin heavy chains (CHC) and three tightly associated light chains (CLC), assemble into a polygonal lattice, which helps to deform the overlying plasma membrane into a coated pit. Heterotetrameric AP2 complexes are targeted to the plasma membrane by the α-adaptin subunits, where they mediate clathrin assembly through the β2-subunit, and interact directly with sorting motifs on cargo molecules through their μ2 subunits. Dynamin is a multidomain GTPase that is recruited to the necks of coated pits and assemble into a

spiral, resulting in the scission and release of CCVs. A subsequent uncoating reaction recycles the coat constituents for reuse. Adapted from Conner and Schmid 2003.

1.5.2 Endocytosis-independent functions of clathrin

Many proteins possess multiple and sometimes unexpected functions. This is also the case of clathrin, especially its heavy chain (CHC). In addition to the well-characterized function in endocytosis, moonlighting functions of CHC in the nucleus have been reported. Ten years ago, Okamoto et al. have found that clathrin associates with mitotic spindle during mitosis as formation of clathrin-coated vesicles is shut-down in cells undergoing mitosis (Okamoto 2006). In 2005, Royle et al. have demonstrated for the first time that clathrin stabilizes fibers of the mitotic spindle to assist congression of chromosomes (Royle, Bright et al. 2005). They observed that mitosis is prolonged due to destabilized kinetochore fibers and defective congression of chromosomes when CHC is depleted by RNAi (Royle, Bright et al. 2005). In addition, Enari et al have shown that nuclear CHC binds to the tumor suppressor p53 to enhance p53-dependent transactivation, which promotes p53 target gene expression (Enari, Ohmori et al. 2006).

1.5.3 Regulation of NF-κB signalling pathway by clathrin-binding proteins of endocytic pathway

Activation of NF-κB is mediated by sequential phosphorylation and activation of signalling proteins involved in the NF-κB pathways upon receptor stimulation (*e.g.*TNFR-RIP-TAK1-IKK, TLR/IL-1R-IRAK-TAK1-IKK, and Nod1-RIP2-TAK1-IKK). However, how

those kinases remain inactive in resting cells is largely unknown. Protein-protein interaction studies have revealed that several endocytic proteins play additional function as inhibitors of NF-κB signalling pathways. Tom1 (target of Myb1) has been shown to interact with Tollip (Toll-interacting protein), forming a complex to regulate endosomal trafficking of ubiquitinated proteins such as IL-1R (Brissoni, Agostini et al. 2006). In addition, Tollip forms a complex with IRAK (IL-1R-associated kinase) and blocks phosphorylation of IRAK, which prevents IKK and NF-κB activation upon stimulation of IL-1R but not TNFR (Burns, Clatworthy et al. 2000). Tom1 is proposed to be a common negative regulator of signalling pathways induced by IL-1β and TNFα (Yamakami and Yokosawa 2004). Recently, it was shown that Tom1 also inhibits NF-κB activation upon TLR2/4 stimulation (Oglesby, Bray et al. 2010). Tom1 can also bind to CHC via a typical clathrin binding motif (DLIDMG) and ubiquitin chains (Yamamoto, Verma et al. 2003). However, the connection between Tom1/Tollip and CHC in the regulation of NF-κB has not been addressed.

β-arrestins (β-arrestin 1 and β-arrestin 2) were initially known as negative regulators of G-protein-coupled receptors (GPCRs)-mediated signalling (Reiter and Lefkowitz 2006). Activation of GPCRs such as β2-adrenergic receptor promotes the recruitment of cytosolic β-arrestins to the phosphorylated (activated) receptor. This uncouples the receptor from G proteins and promotes the receptor internalization, thus causing desensitization (Claing, Laporte et al. 2002). However, new roles of β-arrestins in MAPK signaling, and NF-κB and p53-mediated transcriptional regulation have been discovered (Gao, Sun et al. 2004; Lefkowitz and Whalen 2004; Shenoy, Drake et al. 2006). β-arrestins-IκBα interaction was identified in yeast two-hybrid assays (Witherow, Garrison et al. 2004). Moreover,

stimulation of β2-adrenergic receptor in HEK 293, HeLa and COS-7 cells significantly increases the amount of β-arrestin 2, which is then associated with IκBα (Gao, Sun et al. 2004). The interaction with β-arrestin 2 prevents phosphorylation and degradation of IκBα and thus attenuates activation of NF-κB and transcription of NF-κB target genes (Gao, Sun et al. 2004; Witherow, Garrison et al. 2004). Functional relevance of β-arrestins regulation of NF-κB was further confirmed in the NF-κB activating signalling pathways. Luan et al. demonstrated that β-arrestin 2 can function as a suppressor of ultraviolet-induced NF-κB activation through a direct interaction with IκBα (Luan, Zhang et al. 2005). In addition, Wang et al. discovered that β-arrestins modulate TLR/IL-1R-mediated NF-κB signalling through their interaction with TRAF6, preventing autoubiquitination of TRAF6 (Wang, Tang et al. 2006). However, different from the β-arrestins- IκBα interaction, stimulation of β2-adrenergic receptor has no effect on the interaction of β-arrestins and TRAF6, suggesting that β-arrestins-TRAF6 interaction is regulated by IL1R independently of GPCR stimulation (Wang, Tang et al. 2006). β-arrestins can bind to clathrin through the C-terminal clathin binding domain (Krupnick, Goodman et al. 1997).

1.6 Aim of the study

Nuclear factor-kappaB (NF-κB) is a cytosolic transcription factor in resting cells and translocates into the nucleus and becomes active in response to pro-inflammatory stimuli and bacterial infection. The IKK complex contains two catalytic subunits IKKα and IKKβ and is essential for the activation of NF-κB through phosphorylation and degradation of the inhibitor of NF-κB (IκBα). IKKα and IKKβ are structurally similar but functionally distinct each other, with IKKα being important for lymphocyte organogenesis but IKKβ being critical for inflammation and innate immunity.

The primary goal of my Ph.D. thesis research was to identify signaling proteins important for NF-κB regulation in resting cells and in response to *Shigella flexneri.* Initial efforts have been focused on an RNAi screen. Following the screen, special aims of the follow-up studies were:

1) To reveal an unknown function of IKKα in inflammation and innate immunity

2) To investigate an endocytosis-independent function of clathrin heavy chain (CHC) in the regulation of basal NF-κB activation.

CHAPTER 2

IKKα contributes to canonical NF-κB activation downstream of Nod1-mediated peptidoglycan recognition

(MANUSCRIPT IN PRESS)

IKKα contributes to canonical NF-κB activation downstream of Nod1-mediated peptidoglycan recognition

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Running title: IKKα in NF-κB activation

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2.1 ABSTRACT

Background: During pathogen infection, innate immunity is initiated via the recognition of microbial products by pattern recognition receptors and the subsequent activation of transcription factors that upregulate proinflammatory genes. By controlling the expression of cytokines, chemokines, anti-bacterial peptides and adhesion molecules, the transcription factor nuclear factor-kappa B (NF-κB) has a central function in this process. In a typical model of NF-κB activation, the recognition of pathogen associated molecules triggers the canonical NF-κB pathway that depends on the phosphorylation of Inhibitor of NF-κB (IκB) by the catalytic subunit IκB kinase β (IKKβ), its degradation and the nuclear translocation of NF-κB dimers.

Methodology: Here, we performed an RNA interference (RNAi) screen on *Shigella flexneri*-induced NF-κB activation to identify new factors involved in the regulation of NF-κB following infection of epithelial cells by invasive bacteria. By targeting a subset of the human signaling proteome, we found that the catalytic subunit IKKα is also required for complete NF-κB activation during infection. Depletion of IKKα by RNAi strongly reduces the nuclear translocation of NF-κB p65 during *S. flexneri* infection as well as the expression of the proinflammatory chemokine interleukin-8. Similar to IKKβ, IKKα contributes to the phosphorylation of IκBα on serines 32 and 36, and to its degradation. Experiments performed with the synthetic Nod1 ligand L-Ala-D-γ-Glu-meso-diaminopimelic acid confirmed that IKKα is involved in NF-κB activation triggered downstream of Nod1-mediated peptidoglycan recognition.

Conclusions: Taken together, these results demonstrate the unexpected role of IKKα in the canonical NF-κB pathway triggered by peptidoglycan recognition during bacterial infection.

In addition, they suggest that IKKα may be an important drug target for the development of treatments that aim at limiting inflammation in bacterial infection.

2.2 INTRODUCTION

During pathogen infection, structurally conserved microbial molecules are recognized by germline-encoded pathogen recognition receptors (PRRs) that function as sensors for non-self detection and initiate innate immunity (Takeuchi and Akira ; Medzhitov 2007). PRRs include transmembrane proteins such as Toll-like receptors and C-type lectin receptors, as well as cytoplasmic proteins such as retinoic acid-inducible gene (RIG)-I-like receptors and NOD-like receptors (Blasius and Beutler ; Franchi, Park et al. 2008; Kawai and Akira 2008). They are expressed in macrophages and dendritic cells but also in various non-professional immune cells including epithelial and endothelial cells. PRRs recognize a large variety of pathogen associated molecular patterns (PAMPs) from both extracellular and intracellular pathogens including lipopolysaccharide, peptidoglycan, lipoproteins, dsRNA, ssRNA, CpG-DNA and flagellin (Rasmussen, Reinert et al. 2009). Signaling pathways of PAMP recognition converge into the activation of the pleiotropic transcription factor nuclear factor-kappa B (NF-κB) that, in the context of innate immunity, regulates the expression of proinflammatory genes encoding cytokines, chemokines, anti-bacterial peptides and adhesion molecules (Beutler 2009). The mammalian NF-κB family consists of the members RelA/p65, RelB, c-Rel, p50 (NF-κB1) and p52 (NF-κB2) (Hayden and Ghosh 2004). All five proteins share a Rel homology domain and form homo- and heterodimers that regulate transcription by binding to κB sites in promoters or enhancers of target genes. In unstimulated cells, most of the NF-κB dimers are sequestrated in the cytoplasm by the proteins of the Inhibitor of NF-κB (IκB) family whose prototype is IκBα. In the canonical pathway of NF-κB activation triggered by most stimuli including bacterial and viral infection, cytokines and stress-induced responses, phosphorylation of IκBα on Serine 32

and Serine 36 residues by the IκB kinase (IKK) complex is a decisive regulatory step (Solt and May 2008). The IKK complex is comprised of three subunits: two catalytic subunits, IKKα and IKKβ, and the regulatory scaffold component NF-κB essential modulator (NEMO). The respective contribution of IKKα and IKKβ in the phosphorylation of IκBα is unclear. Although it is generally accepted that IKKβ is critical for IκBα phosphorylation through the canonical pathway, two recent reports demonstrate the equal importance of IKKα for the activation of NF-κB by the inflammatory cytokines interleukin-1 (IL-1) in mouse embryonic fibroblasts and tumor necrosis factor alpha (TNFα) in HeLa cells (Adli, Merkhofer et al. ; Solt, Madge et al. 2007). The phosphorylation of IκBα is followed by its rapid polyubiquitination and subsequent degradation by the 26S proteasome complex (Gilmore 2006). The release of NF-κB with unmasked nuclear localization sequence leads then to the translocation of the transcription factor to the nucleus where it regulates gene expression (Hayden and Ghosh 2008).

Although the role of NF-κB is central to many pathways triggered by pathogen recognition, the molecular processes that govern its activation are only partially elucidated. In particular, the mechanisms triggered by the detection of invasive bacteria such as the pathogen *Shigella flexneri* remain largely uncharacterized. *S. flexneri* makes use of a type III secretion (T3S) apparatus to locally rearrange the host actin cytoskeleton and penetrate into intestinal epithelial cells (Schroeder and Hilbi 2008). Once internalized, bacteria multiply in the host cytoplasm and use actin-based motility to spread to adjacent epithelial cells. During infection, massive inflammation is observed in colonic mucosal tissues (Islam, Veress et al. 1997). In infected epithelial cells, intracellular bacteria release peptidoglycan-derived peptides that are specifically recognized by Nod1 (Girardin, Boneca

et al. 2003). Upon ligand binding, Nod1 homo-dimerizes and recruits the downstream kinase RICK/RIPK2 through heterologous caspase-recruitment domain interactions (Inohara, Koseki et al. 2000). This converges to the sequential recruitment and activation of the TAK1/TAB1/TAB2 and IKKα/IKKβ/IKKγ complexes, the nuclear translocation of NF-κB and the upregulation of proinflammatory genes encoding for cytokines and chemokines, including interleukin-8 (IL-8) and TNFα (Sansonetti, Arondel et al. 1999). The chemokine IL-8 recruits polymorphonuclear cells to the site of infection and therefore contributes to contain the dissemination of bacteria within the intestinal tissue. Interestingly, *S. flexneri* uses the T3S apparatus to secrete several effectors that alter multiple signaling pathways in infected cells and reduce the expression of proinflammatory genes (Ogawa, Handa et al. 2008). Among others, the effector OspF suppresses the expression of IL-8 by dephosphorylating the MAP kinases p38 and ERK in the nucleus of infected cells (Arbibe, Kim et al. 2007; Li, Xu et al. 2007), thereby impairing the phosphorylation of Histone H3, a process that regulates the access of chromatin to transcription factors.

Here, we performed an RNA interference (RNAi) screen on *S. flexneri*-induced NF-κB activation to identify new factors involved in the regulation of NF-κB following infection of epithelial cells by invasive bacteria. By targeting a subset of the human signaling proteome, we identified IKKα as a protein required for *S. flexneri*-induced NF-κB nuclear translocation and IL-8 secretion in HeLa cells. This result was unexpected because, except for IL-1 and TNFα (Adli, Merkhofer et al. ; Solt, Madge et al. 2007), it is generally accepted that IKKβ is the component of the IKK complex involved in the canonical pathway of NF-κB activation. Depletion of IKKα or IKKβ indicated that *S. flexneri*-induced NF-κB activation in HeLa cells requires indeed both catalytic subunits.

We further characterized the role of IKKα and found that, during *S. flexneri* infection, IKKα was required for the phosphorylation of IκBα on serines 32 and 36, and for its degradation. Experiments performed with the synthetic Nod1 ligand L-Ala-D-γ-Glu-meso-diaminopimelic acid (Tri-DAP) indicated that IKKα was involved in Nod1-mediated signaling pathway of NF-κB activation. Taken together, these results show that, although Nod1 signaling triggers the canonical pathway of NF-κB activation, both IKKα and IKKβ are required for full NF-κB activation.

2.3 MATERIALS AND METHODS

Antibodies and reagents

Antibodies against NF-κB p65, IκBα and IKKα were obtained from Santa Cruz Biotechnology (Santa Cruz, USA) whereas the anti-actin was from Chemicon (Billerica, USA) and the anti-phospho-IκBα was from Cell signaling technology (Beverly, USA). The anti-mouse IgG-Cy5 was obtained from Zymed (San Francisco, USA) and the anti-rabbit IgG-HRP and anti-mouse IgG-HRP from GE Healthcare (Pittsburgh, USA). Hoechst and FITC-phalloidin were from Invitrogen (Carlsbad, USA), TNFα from R & D systems (Minneapolis, USA).

Cell culture and transfection

HeLa cells were maintained in Dulbecco's modified Eagle's medium (high glucose) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin at 37 °C in 10% CO_2. HeLa cells were transfected with siRNAs and DNA plasmids using Lipofectamine 2000 (Invitrogen, Carlsbad, USA) and jetPEI (Poly plus transfection, Illkirch, France), respectively. siRNAs ON-TARGETplus SMARTpool targeting IKKα (#L-003473-00-005) and ON-TARGETplus siCONTROL (Dharmacon, Dallas, USA) were used in all our study except for the experiments where IKKα, IKKβ and NEMO were silenced in parallel. In this case, all siRNAs were from Qiagen (Valencia, CA, USA).

In vitro diced siRNA library

An *in vitro* diced siRNA library targeting 132 genes coding for a subset of the signaling proteome was generated as previously described (Liou, Kim et al. 2005; Brandman, Liou et al. 2007; Galvez, Teruel et al. 2007). Briefly, for each gene, a 600 base pair cDNA was generated by PCR from a total cDNA library. An additional set of nested primers was used to add T7 promoters at both ends of the final cDNA fragment. Nested PCR products were subject to *in vitro* transcription, dicing, and purification to produce gene specific siRNA pools. Dicing was performed with the turbo dicer siRNA generation kit from Genlantis (San Diego, USA). The concentration of all siRNA pools was normalized.

Bacterial strains

The *S. flexneri* strains M90T wild-type and the *icsA* (*virG*) deletion mutant (Δ*virG*) were generously provided by Dr. P. Sansonetti (Institut Pasteur, Paris, France). All strains were transformed with the pMW211 plasmid to express the DsRed protein under control of a constitutive promoter. The pMW211 plasmid was a generous gift from Dr. D. Bumann (Biozentrum, University of Basel, Switzerland). The Δ*ospF* deletion mutant used in IL-8 expression experiments, was generated from the Δ*virG* mutant by allelic exchange using a modification of the lambda red-mediated gene deletion (Datsenko and Wanner 2000). Briefly, the genes for lambda red recombination were expressed from the pKM208 plasmid (Murphy and Campellone 2003). The chloramphenicol resistance cassette (cat) of the pKD3 plasmid was amplified using the primers listed in Table 1. After DpnI digestion, the PCR product was electroporated into the Δ*virG* mutant. Recombinants were selected on TSB plates containing 5 or 10 μg ml^{-1} chloramphenicol. The cat cassette was removed by

transformation of pCP20 and incubation at 30°C on TSB plates containing 100 μg ml^{-1} ampicillin (Datsenko and Wanner 2000). Single colonies were screened by PCR.

Table 1. Oligonucleotide primers used to generate the Δ*ospF* mutant.

Mutant	Forward	Reverse
Δ*ospF*	ATTCTATTATATAGATAAAATATCT CCTGCAAAAGATACGGGTATTTT TGTGTAGGCTGGAGCTGCTTCG	TCAAAAGTTCGATGTTCCACCACAT CGACCGTAGAAGAGATGAGATAGTA CATATGAATATCCTCCTTAG

Infection assay

Bacteria were routinely grown in tryptic soy broth (TSB) medium, used in exponential growth phase, and treated with poly-L-lysine prior infection. HeLa cells, seeded in 96-well plates, were serum starved for 30 min and infected with *S. flexneri* at a multiplicity of infection (MOI) of 10. Immediately after adding bacteria, the plates were centrifuged for 5 min at 2000 rpm and placed at 37°C for 30 min. Extracellular bacteria were killed by addition of gentamycin (50 μg/ml).

Immunofluorescence

Cells were fixed with 4% PFA for 6 min and permeabilized in 0.5% Triton X-100 for 10 min. They were, then, incubated with a mouse monoclonal anti-p65 antibody (1 μg/ml) overnight at 4 °C and stained with a Cy5-conjugated secondary antibody and Hoechst (10 μg/ml) for 40 min at room temperature.

siRNA screen of *S. flexneri*-induced p65 nuclear translocation

The *in vitro* diced siRNA library was screened on *S. flexneri*-induced p65 translocation assay in a 96-well format. The firefly *luciferase* (GL3) siRNA was used as a non-silencing negative control as described previously (Liou, Kim et al. 2005; Brandman, Liou et al. 2007; Galvez, Teruel et al. 2007). siRNA pools against Nod1, RIPK2 and Src were used as positive controls. The screen was performed three times in duplicate as follows. Three thousand HeLa cells per well were transfected by reverse transfection with the individual 132 siRNA pools in 96-well plates. After 48 hours, cells were infected with DsRed *S. flexneri* at MOI of 10 for 90 min and then fixed, permeabilized, and stained for p65, F-actin, and DNA. Images were acquired at 12 random sites of each well using the automated ImageXpress microscope (Molecular devices, Sunnyvale, USA). At each site, images at 360 nm, 480 nm, 594 nm, 640 nm were acquired to visualize Hoechst, Phalloidin, DsRed *S. flexneri* and p65, respectively. The nuclear localization of p65 was automatically quantified by using the Enhanced-Translocation module of MetaXpress (Molecular devices, Sunnyvale, USA). Briefly, the Hoechst staining was used as a mask to automatically identify nuclei in the p65 staining image. The cytoplasmic area was defined by a ring around each nucleus. For each cell, the ratio of p65 intensity in the nucleus and in the cytoplasmic ring defined as the Nuc/Cyt p65 ratio was calculated and averaged over several thousands of cells per well. The results of the screen were expressed as individual scores. The score of a particular gene represents the fold standard deviation from the mean of the GL3 control wells. A negative or a positive sign was assigned to the score when the Nuc/Cyt p65 ratio was lower or higher than the GL3 control ratio, respectively.

Enzyme- linked Immunosorbent Assay (ELISA)

IL-8 secretion was measured by ELISA in the supernatant of HeLa cells 6 hours post infection. Cell-free supernatants from triplicate wells were analyzed for their IL-8 content using a commercial ELISA kit (BD Pharmingen, San Jose, USA).

Western Blot Analysis

HeLa cells were transfected with siRNAs in a 6-well plate. 72 hours post transfection, cells were lysed in Phosphosafe Extraction Buffer (Novagen, Darmstadt, Germany) supplemented with 1x protease inhibitor cocktail (Calbiochem, Darmstadt, Germany). Protein concentration was measured using the bicinchonic acid (BCA) kit (Pierce, Rockford, USA). Equal amounts of proteins were resolved by SDS-PAGE and transferred to Hybond C-Extra membrane (Amersham Bioscience, Pittsburgh, USA) for immunoblotting with individual antibodies. Primary antibodies were detected using horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibodies, and visualized with the ECL system (Pierce). Quantification of the blots was performed using the densitometry feature of Photoshop.

Tri-DAP treatment

Cells were serum starved 30 min before treatment with L-Ala-γ-D-Glu-mesoDAP (Tri-DAP). Tri-DAP treatment was performed by calcium phosphate transfection using 0.4 μg/ml Tri-DAP final concentration for 90 min. Then cells were fixed with 4% PFA and analyzed by immunofluorescence as described above.

Statistical analysis

Data are expressed as mean ± standard deviation calculated from the number of replicates specified in figure legends. p values were calculated with a two-tailed two-sample equal variance t-test.

2.4 RESULTS

2.4.1 RNAi screen identifies the role of IKKα in the nuclear translocation of NF-κB p65 during infection of epithelial cells by *S. flexneri*

Over the past 10 years, RNAi has been an essential tool to study gene function in mammalian cells (Liou, Kim et al. 2005; Pelkmans, Fava et al. 2005; Zhang, Liu et al. 2009). Using *S. flexneri*-induced NF-κB activation in HeLa cells as a model system, we performed an image-based RNAi screen to identify new proteins involved in the molecular mechanisms that control NF-κB activation following pathogen recognition. The nuclear localization of NF-κB p65 visualized by anti-p65 immunofluorescence microscopy was used as readout for NF-κB activation. In uninfected cells, p65 was mainly localized in the cytoplasm (Figure 2.1A, left panel). In contrast, following infection with dsRed-expressing *S. flexneri,* a strong nuclear translocation of p65 was observed (Figure 2.1A, right panel). To validate the RNAi approach, silencing of the intracellular pattern recognition receptor Nod1 involved in *S. flexneri* recognition was tested. HeLa cells were transfected with pools of *in vitro* diced small interference RNA (siRNA) targeting Nod1 or the firefly *Luciferase* used as control (GL3). After 48 hours, cells were infected with *S. flexneri*, stained for p65 and DNA with an anti-p65 antibody and Hoechst, respectively. Visual inspection of images showed that *S. flexneri*-induced p65 nuclear translocation was suppressed in Nod1-depleted cells (Figure 2.1B). This observation was quantified by measuring for each cell the ratio of p65 intensity in the nucleus and in the cytoplasm by automated image processing (Figure 2.1C). The Hoechst staining was used to automatically identify nuclei whereas the cytoplasmic area was defined by a ring around each nucleus. Quantification of the Nucleus/Cytoplasm p65 intensity ratio (Nuc/Cyt p65 ratio) confirmed that the depletion of

Nod1 inhibited the nuclear translocation of p65 induced during infection (Figure 2.1D), and therefore, that the RNAi approach was suitable to identify new proteins involved in the activation of NF-κB during *S. flexneri* infection of epithelial cells.

An *in vitro* diced siRNA library targeting 132 genes from the human signaling proteome was screened on *S. flexneri*-induced p65 nuclear translocation as described in Materials and Methods. For each gene, a score representing the fold standard deviation from the mean of GL3 control wells was calculated (Figures 2.1E and Table S1). A negative or a positive sign was attributed to the score when the Nuc/Cyt p65 ratio was lower or higher than the GL3 ratio, respectively. As expected, Nod1 and RIPK2, two key proteins involved in *S. flexneri*-induced NF-κB activation (Girardin, Tournebize et al. 2001; Girardin, Boneca et al. 2003), as well as Src, a tyrosine kinase required for bacterial entry into cells (Dehio, Prevost et al. 1995), obtained strong negative scores (Figures 2.1E and Table S1). Using an arbitrarily determined cut off score value of +/- 3.5, the proteins CHUK/IKKα, FGD3, Arf1, GIT1, Rab23, ARHN, RabGGTA, HIPK2 and CLTC were classified as hits (Figures 2.1E and Table S1). The identification of IKKα, also known as CHUK, was unexpected as peptidoglycan recognition via Nod1 triggers the canonical NF-κB pathway, and was, therefore, thought to be exclusively dependent on IKKβ and NEMO. The contribution of IKKα in the mechanisms that control NF-κB activation following *S. flexneri* infection was then further explored.

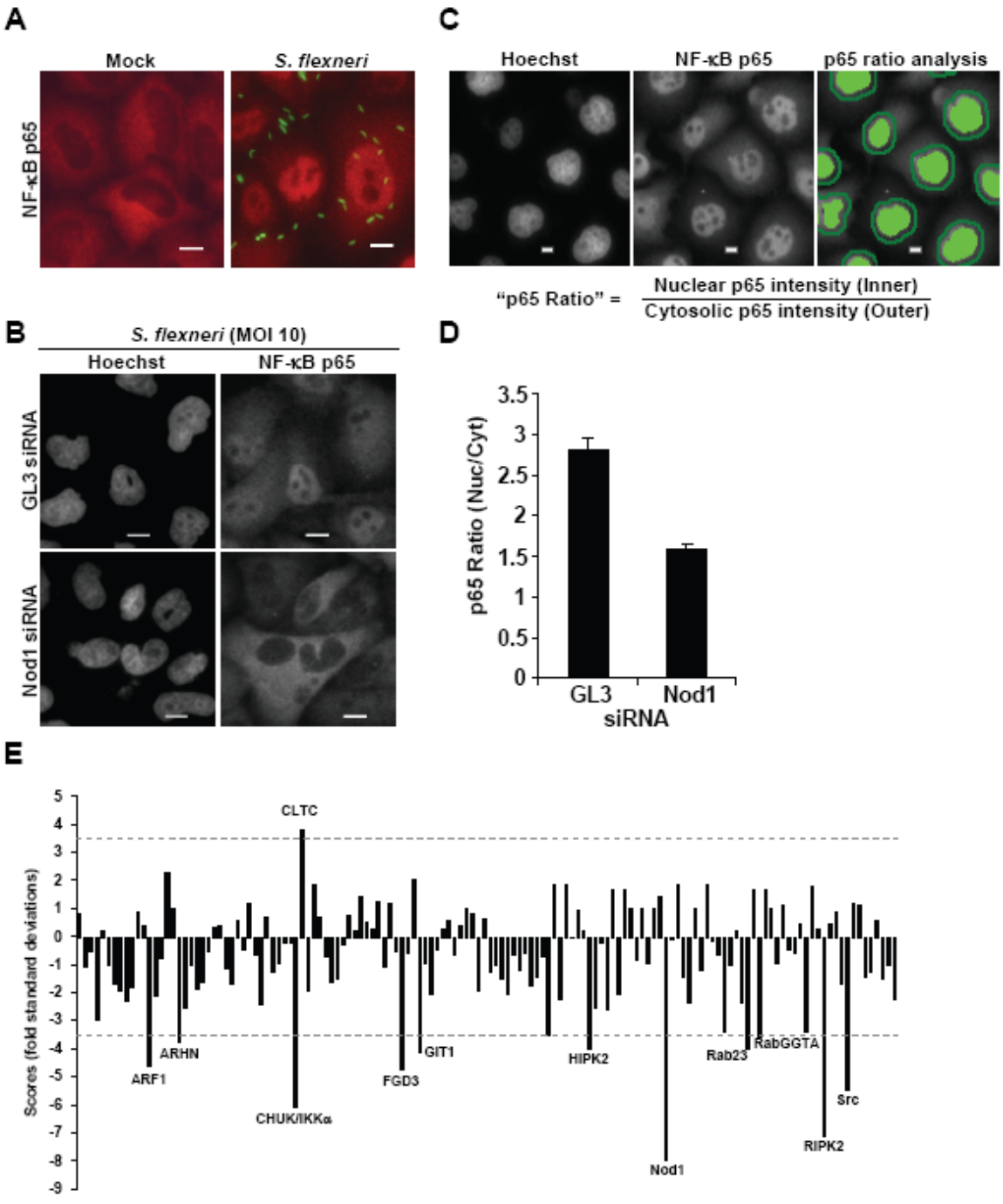

Figure 2.1. **RNAi screen identifies the role of IKKα in p65 nuclear translocation during *S. flexneri* infection**

(**A**) NF-κB p65 translocates to the nucleus in response to *S. flexneri* infection. HeLa cells were left untreated (Mock) or infected with dsRed-expressing *S. flexneri* for 60 minutes at MOI=10. NF-κB p65 localization was visualized by immunofluorescence microscopy. An overlay image is shown for *S. flexneri* (green) and p65 (red). (**B**) Depletion of Nod1 by RNAi inhibits the nuclear

translocation of NF-κB p65 in response to *S. flexneri* infection. HeLa cells were transfected with GL3 or Nod1 siRNA and infected with *S. flexneri* for 60 minutes at MOI=10. p65 and DNA were visualized by anti-p65 and Hoechst staining, respectively. (**C**) Images illustrating the quantification of the p65 ratio. The Hoechst staining was used as a mask to automatically identify the nuclei in the p65 staining image. The cytoplasmic area was defined by a ring surrounding each nucleus. Scale bars, 10 μm (**D**) Quantification of the p65 ratio in control and Nod1-depleted cells following infection by *S. flexneri*. Results represent the mean ± SD of 12 images; graph representative of 3 independent experiments. (**E**) RNAi screen of *S. flexneri*-induced p65 nuclear translocation in HeLa cells. Scores are fold standard deviations from the mean of GL3 control wells. Dashed lines represent scores of ± 3.5 (See Materials and Methods for details).

2.4.2 Depletion of IKKα inhibits *S. flexneri*-induced p65 nuclear translocation and IL-8 expression without affecting bacterial invasion

First, to validate the role of IKKα in *S. flexneri*-induced p65 nuclear translocation, we tested a pool of four synthetic IKKα siRNAs that had no overlapping sequences with the *in vitro* diced siRNAs used in the screen. In conditions where around 90% of endogenous protein was depleted (Figure 2.2A), a massive reduction of p65 nuclear translocation was observed (Figures 2.2B and 2.2C). This result was not explained by a reduction of bacterial uptake as the number of internalized bacteria was similar in control and in IKKα-depleted cells (Figure 2.2B). Taken together, these results indicated that IKKα is required for the activation of NF-κB during infection of epithelial cells by *S. flexneri.*

During infection, NF-κB positively regulates the expression of multiple proinflammatory genes (Pedron, Thibault et al. 2003). In particular, it induces the expression of the chemokine IL-8, which recruits PMNs on site of infection, and thereby limits the spread of bacterial invasion within the intestinal tissue (Sansonetti, Arondel et al.

1999). To test whether IKKα contributed to the upregulation of IL-8 expression during infection, we measured by ELISA the secretion of IL-8 in the supernatant of control and IKKα-depleted HeLa cells six hours post infection. To increase the amount of IL-8 produced in response to infection, cells were infected with a mutant of *S. flexneri* deleted for the type III effector OspF (Δ*ospF*) that dampens inflammation signaling by dephosphorylating p38 in the nucleus of infected cells (Arbibe, Kim et al. 2007). Consistent with NF-κB data, a reduction of IL-8 secretion was observed in response to *S. flexneri* Δ*ospF* infection when cells were depleted for IKKα (Figure 2.2D), showing that IKKα is involved in the signaling pathways that control the expression of a critical inflammatory chemokine during bacterial infection.

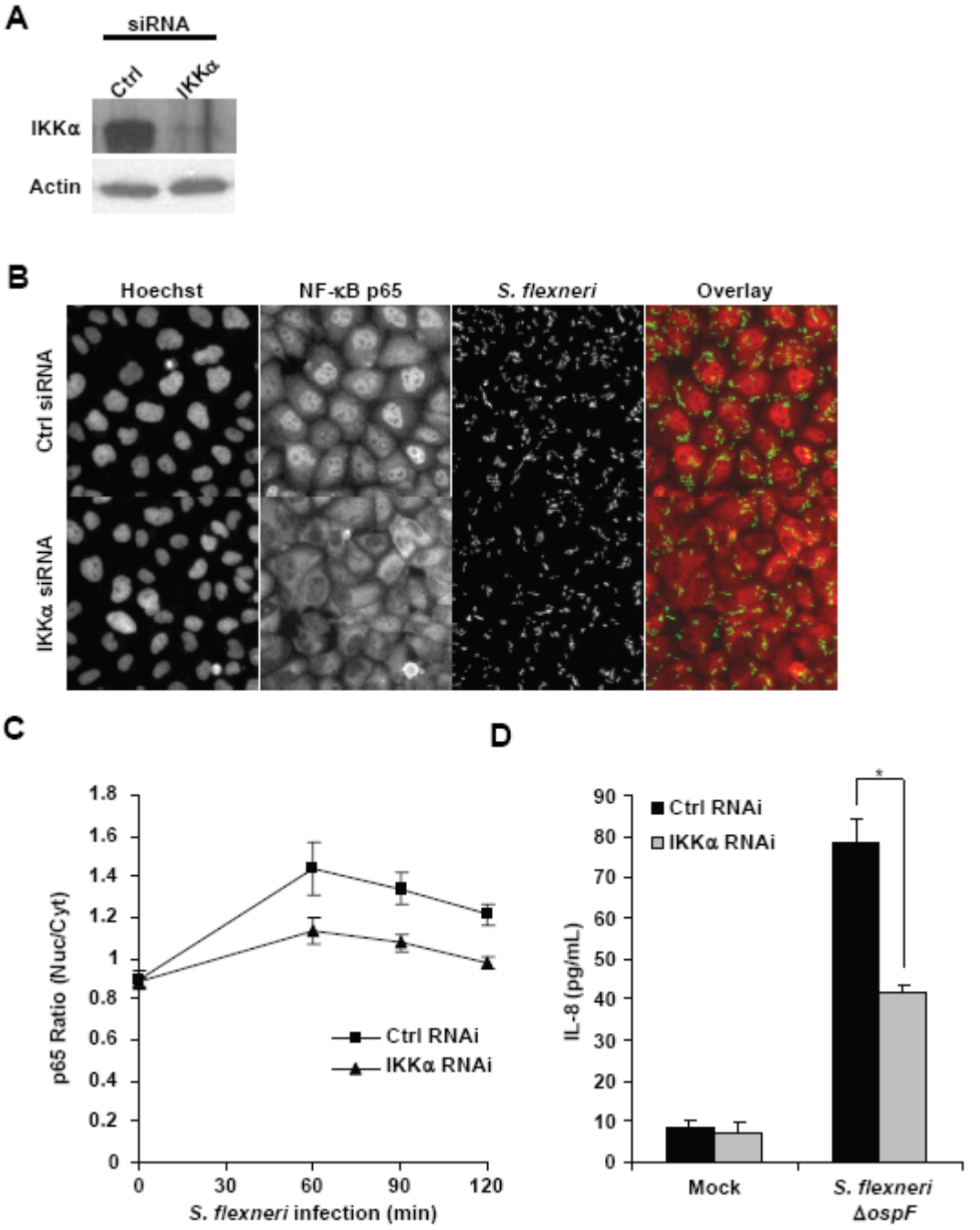

Figure 2.2. **Depletion of IKKα inhibits *S. flexneri*-induced p65 nuclear translocation and IL-8 expression without affecting bacterial invasion**

(**A**) IKKα expression is effectively reduced by RNAi. HeLa cells were transfected with the indicated siRNAs. Western blots were performed on cell lysates. Actin levels are shown as a loading control. (**B**) Depletion of IKKα inhibits *S. flexneri*-induced p65 nuclear translocation and IL-8 expression without affecting bacterial invasion. HeLa cells were transfected with control or

IKKα siRNA and infected with *S. flexneri* for 60 minutes at MOI=10. An overlay is shown for *S. flexneri* (green) and p65 (red), right panel. (**C**) Time course of p65 nuclear translocation during *S. flexneri* infection of control and IKKα-depleted cells. Results represent the mean ± SD of 12 images; graph representative of 2 independent experiments. (**D**) *S. flexneri*-induced IL-8 secretion is impaired by the depletion of IKKα. IL-8 secretion was measured by ELISA in the supernatant of control or IKKα-depleted HeLa cells left untreated (Mock) or infected with *S. flexneri* Δ*ospF*. Results represent the mean ± SD of 6 wells; graph representative of 3 independent experiments, *p=0.006.

2.4.3 Both IKKα and IKKβ contribute to the phosphorylation and the degradation of IκBα during *S. flexneri* infection

It is generally believed that IKKβ and NEMO are the two subunits of the IKK complex involved in the canonical pathway of NF-κB activation. To test the contribution of all IKK subunits in the activation of NF-κB during infection of epithelial cells by *S. flexneri*, we monitored the localization of p65 in cells depleted for IKKα, IKKβ or NEMO. As shown in Figure 2.3A, the depletion of either of these proteins reduced the nuclear translocation of p65, indicating that both catalytic subunits IKKα and IKKβ, as well as the scaffolding function of NEMO were required to fully activate the NF-κB pathway during infection. IKKβ regulates the canonical pathway of NF-κB activation by phosphorylating IκBα at positions serine 32 and 36, thereby inducing its polyubiquitination and subsequent degradation (Solt and May 2008). To test whether IKKα regulated NF-κB via a similar mechanism, we monitored the phosphorylation of IκBα at serines 32 and 36 (pIκBα) and its degradation following infection of HeLa cells by *S. flexneri*. Whereas massive phosphorylation and degradation of IκBα were observed in control cells following infection, these two processes were strongly reduced after IKKα knockdown (Figure 2.3B),

indicating that IKKα largely contributes to the phosphorylation and degradation of IκBα during infection by *S. flexneri*.

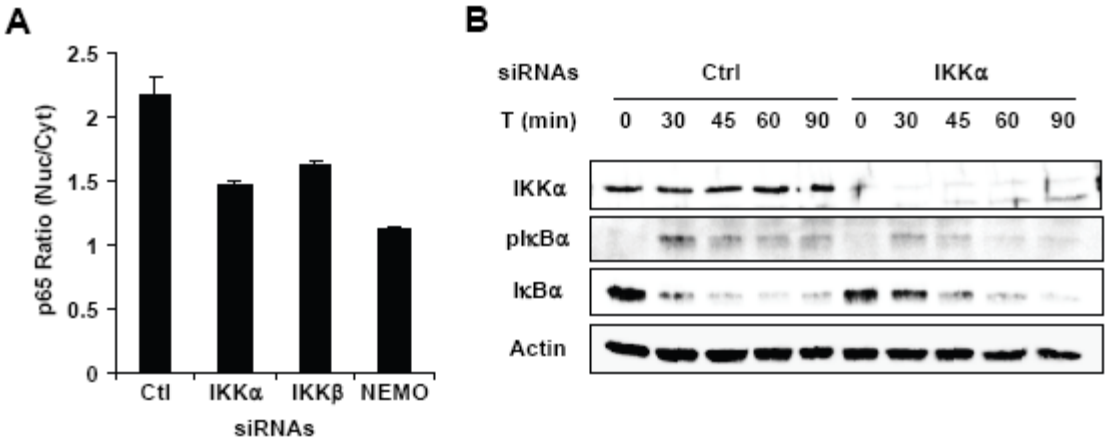

Figure 2.3. **Both IKKα and IKKβ catalytic subunits contribute to NF-κB activation during infection by *S. flexneri*.**

(**A**) Depletion of IKKα, IKKβ or NEMO reduces the translocation of p65 during *S. flexner*i. *S. flexneri*-induced p65 translocation was analyzed in HeLa cells transfected with control, IKKα, IKKβ and NEMO siRNAs. Results represent the mean ± SD of 12 images; graph representative of 2 independent experiments. (**B**) IKKα is involved in the phosphorylation and the degradation of IκBα during *S. flexneri* infection. Control or IKKα-depleted HeLa cells were left untreated or infected with *S. flexneri* for the indicated periods at MOI=10. Levels of IKKα, pIκBα and IκBα were monitored by western blots performed on cell lysates. Actin levels are shown as a loading control.

2.4.4 IKKα is involved in Nod1-mediated peptidoglycan recognition

During *S. flexneri* infection, the activation of NF-κB is initiated by the recognition of peptidoglycan fragments via the intracellular receptor Nod1 (Girardin, Boneca et al. 2003). To specifically examine the implication of IKKα in Nod1-mediated signaling, the effect of IKKα depletion was directly tested in cells exposed to the Nod1 ligand Tri-DAP. Whereas Tri-DAP treatment induced a clear nuclear translocation of p65 in control cells, this translocation was severely impaired in IKKα-depleted cells (Figures 2.4A and 2.4B), demonstrating that IKKα is required for the activation of NF-κB following Nod1-mediated peptidoglycan recognition.

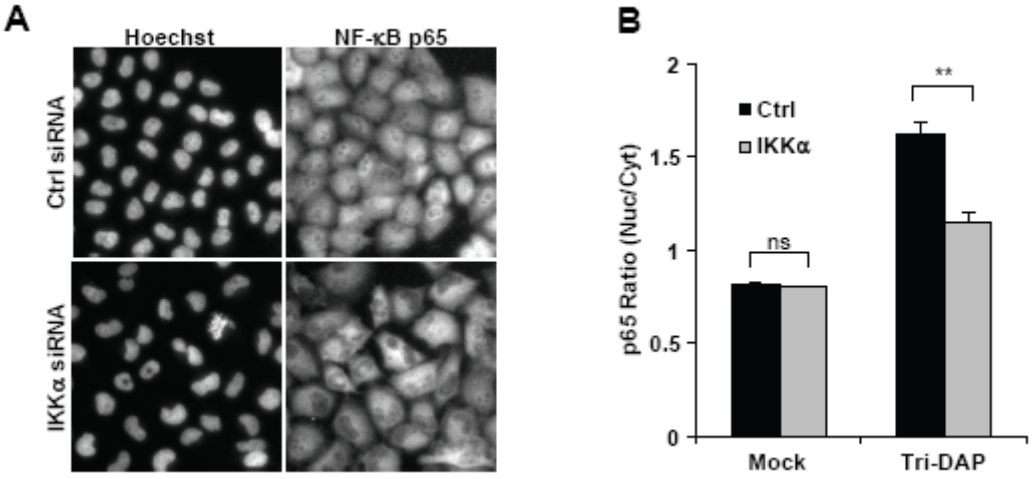

Figure 2.4 IKKα is required for the activation of NF-κB induced by Nod1-mediated peptidoglycan recognition

(**A**) IKKα depletion reduces the activation of NF-κB induced by Nod1-mediated peptidoglycan recognition. Control or IKKα-depleted HeLa cells were treated with Tri-DAP as described in Materials and Methods. After 1 hour, cells were fixed, stained for p65 and DNA with an anti-p65 antibody and Hoechst, respectively. (**B**) Quantification of the effect of IKKα depletion on Tri-DAP-induced p65 nuclear translocation. Results represent the mean ± SD of 12 images; graph representative of 3 independent experiments, **p ≤ 8.6E-4, ns: non-significant.

Table S1. Results of the screen on *S. flexneri*-induced p65 nuclear translocation

Gene	Gene ID	Score	Gene	Gene ID	Score	Gene	Gene ID	Score
CLTC	1213	3.833	RHOB	388	0.260	UBA7	7318	-1.413
ARHGEF7	8874	2.318	IPO7	10527	0.237	HIP1	3092	-1.415
GIPC2	54810	2.009	PTPRO	5800	0.224	PDE7B	27115	-1.428
PRKAG1	5571	1.887	ADCY3	109	0.220	DLG5	9231	-1.464
HIPK3	10114	1.886	DUSP2	1844	0.203	UBE2N	7334	-1.506
DBN1	1627	1.863	HOMER2	9455	-0.056	GRK7	131890	-1.508
HOMER1	9456	1.861	NOD2	64127	-0.125	ARPC3	10094	-1.611
P2RY14	9934	1.845	PRKAG2	51422	-0.144	DGKQ	1609	-1.625
RHOA	387	1.779	AGAP3	116988	-0.210	ADRBK1	156	-1.655
MPP1	4354	1.699	AGAP1	116987	-0.228	SPTB	6710	-1.672
RAC1	5879	1.685	LIN7A	8825	-0.263	FERMT1	55612	-1.717
LIN7C	55327	1.633	DUSP15	128853	-0.297	GUCY2D	3000	-1.751
RAB25	57111	1.626	ADAP1	11033	-0.456	AP2A2	161	-1.799
DUSP6	1848	1.422	RAP1B	5908	-0.509	ARL11	115761	-1.876
NCK2	8440	1.413	ABL1	25	-0.516	CSK	1445	-1.886
EPHB6	2051	1.275	ERN2	10595	-0.545	GRK1	6011	-1.900
ADAP2	55803	1.186	ARPC4	10093	-0.548	ANLN	54443	-1.953
SYK	6850	1.184	GUCY1A2	2977	-0.571	TAB1	10454	-2.043
ERN1	2081	1.146	RAP1GAP	5909	-0.603	GRLF1	2909	-2.060
CAND1	55832	1.138	CCNYL1	151195	-0.605	ARF6	382	-2.136
RAP1A	5906	1.125	GUCA1B	2979	-0.645	HIPK4	147746	-2.224
MTMR4	9110	1.046	ACAP1	9744	-0.678	ZAP70	7535	-2.252
RAC2	5880	1.045	PTEN	5728	-0.698	AP1B1	162	-2.305
PPP2R5E	5529	1.019	DGKG	1608	-0.730	PHIP	55023	-2.366
MTMR2	8898	0.990	HIPK1	204851	-0.739	RAB14	51552	-2.390
ARHGEF9	23229	0.978	ARHGAP25	9938	-0.763	ACAP1	9744	-2.435
NCK1	4690	0.963	MTMR3	8897	-0.855	NUAK1	9891	-2.524
HOMER3	9454	0.912	GIT2	9815	-0.949	LAT	27040	-2.575
SNRK	54861	0.885	AGAP2	116986	-0.950	LIN7B	64130	-2.628
AP2B1	163	0.871	RAC3	5881	-0.954	TNK2	10188	-2.974
AAK1	22848	0.819	MTMR8	55613	-0.958	RGS19	10287	-3.398
DUSP16	80824	0.765	ADCY8	114	-0.989	PTPN14	5784	-3.416
DGKA	1606	0.677	GRK6	2870	-0.990	HIPK2	28996	-3.523
ACAP2	23527	0.675	PTPN18	26469	-0.991	RABGGTA	5875	-3.567
GRK4	2868	0.653	ARL1	400	-1.022	RND2	8153	-3.801
CD2AP	23607	0.616	UBE3A	7337	-1.028	RAB23	51715	-3.979
UBE2D1	7321	0.534	ABI1	10006	-1.061	KIF11	3832	-4.053
EPHA3	2042	0.523	EPS8	2059	-1.062	GIT1	28964	-4.124
RAP2C	57826	0.473	BUB1B	701	-1.132	ARF1	375	-4.664
SHROOM3	57619	0.462	GUCA1B	2979	-1.201	FGD3	89846	-4.722
ARRB2	409	0.401	PRKACB	5567	-1.204	SRC	6714	-5.509
APPL1	26060	0.354	ACAP3	116983	-1.244	CHUK	1147	-6.098
ARRB1	408	0.332	GRK5	2869	-1.281	RIPK2	8767	-7.127
EPHA5	2044	0.291	UBA6	55236	-1.302	NOD1	10392	-7.951

Scores are fold standard deviations from the mean of GL3 control p65 ratios. Gene names are based on the NCBI nomenclature.

2.5 DISCUSSION

Although NF-κB is a transcription factor that has been the subject of intensive research by academic laboratories and the pharmaceutical industry, the complex molecular mechanisms controlling its activation are only partially elucidated. Here we performed an RNAi screen to identify new proteins involved in the mechanisms that control NF-κB activation following pathogen recognition using infection of epithelial cells by the invasive bacterium *S. flexneri* as a model system. The presence of *S. flexneri* in the cytoplasm is recognized via the detection of peptidoglycan by the receptor Nod1 (Girardin, Boneca et al. 2003). This recognition leads to NF-κB activation and the upregulation of proinflammatory genes that orchestrate the host inflammatory response (Pedron, Thibault et al. 2003). The protein IKKα was identified in the screen as a protein required for the activation of NF-κB in response to *S. flexneri* infection. Since bacterial uptake was not affected by the depletion of IKKα, this result indicated that IKKα is involved in the activation of NF-κB in response to infection. The depletion of IKKβ or NEMO also induced a reduction of *S. flexneri*-induced-p65 nuclear translocation, confirming that all subunits were required for the full activation of NF-κB during infection. As the translocation of p65 depends on the degradation of IκBα primed by its phosphorylation on serines 32 and 36, the role of IKKα in these two processes was analyzed. A reduction of IκBα phosphorylation and delayed degradation were observed in cells depleted for IKKα , indicating that similar to IKKβ, IKKα triggers NF-κB activation by inducing the phosphorylation and the degradation of IκBα. Taken together, these results provided new evidence for a role of IKKα in the canonical pathway of NF-κB activation, and were in line with few reports indicating that the dominant model of canonical NF-κB activation based on IKKβ and NEMO is

incomplete. In particular, it has been reported that IKKα is the key subunit responsible for the Receptor activator of NF-κB (RANK)-induced classical NF-κB activation in mammary epithelial cells (Cao, Bonizzi et al. 2001). In addition, Solt et al. showed that IL-1-induced NF-κB activation requires the interaction of IKKα with NEMO and occurs in the absence of IKKβ (Solt, Madge et al. 2007) . Finally, it has been shown recently that both IKKα and IKKβ contribute to IκBα phosphorylation and NF-κB activation in response to TNFα stimulation in HeLa cells (Adli, Merkhofer et al.). For the first time, our results demonstrate that IKKα is also implicated in the canonical pathway of NF-κB activation triggered by bacterial infection. To further demonstrate that pathogen recognition induced NF-κB activation in an IKKα-dependent manner during *S. flexneri* infection, we directly tested whether IKKα was involved in the activation of NF-κB induced by Nod1-mediated peptidoglycan recognition. For this purpose, controls or IKKα-depleted cells were directly stimulated with the purified Nod1 ligand Tri-DAP. The analysis of the p65 nuclear translocation showed that IKKα was required for the full activation of NF-κB in response to Tri-DAP treatment, indicating that IKKα is involved in the molecular mechanism signaling the recognition of peptidoglycan-derived peptides by Nod1.

Following infection of epithelial cells by *S. flexneri*, the activation of NF-κB leads to the upregulation of genes encoding for inflammatory cytokines including IL-8 and TNFα (Pedron, Thibault et al. 2003). To confirm the implication of IKKα in the expression of genes induced by the canonical NF-κB pathway, the deletion of IKKα was tested on *S. flexneri*-induced IL-8 expression. Consistent with NF-κB data, the secretion of IL-8 was reduced when IKKα was depleted, showing that the contribution of IKKα to NF-κB activation has a functional impact on the amplitude of the inflammatory response mounted

in response to bacterial infection. These results suggest that inhibition of IKKα activity may be critical to control inflammation upon bacterial infection. Inhibitors of IKKβ have demonstrated therapeutic benefits in various animal models of inflammatory diseases and are currently in early clinical trials (Ogawa, Azuma et al. ; Ziegelbauer, Gantner et al. 2005; Izmailova, Paz et al. 2007). The data presented in this manuscript suggest that IKKα inhibitors should also be developed and used in combination with IKKβ inhibitors to limit inflammation during bacterial infection or in inflammatory disorders that may involve Nod1 signaling, including asthma, eczema and inflammatory bowel diseases (Reijmerink, Bottema et al. ; Weidinger, Klopp et al. 2005; Le Bourhis, Benko et al. 2007).

2.6 **ACKNOWLEDGMENTS**

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2.7 REFERENCES

Adli, M., E. Merkhofer, et al. "IKKalpha and IKKbeta each function to regulate NF-kappaB activation in the TNF-induced/canonical pathway." PLoS One **5**(2): e9428.

Arbibe, L., D. W. Kim, et al. (2007). "An injected bacterial effector targets chromatin access for transcription factor NF-kappaB to alter transcription of host genes involved in immune responses." Nat Immunol **8**(1): 47-56.

Beutler, B. (2009). "Microbe sensing, positive feedback loops, and the pathogenesis of inflammatory diseases." Immunol Rev **227**(1): 248-263.

Blasius, A. L. and B. Beutler "Intracellular toll-like receptors." Immunity **32**(3): 305-315.

Brandman, O., J. Liou, et al. (2007). "STIM2 is a feedback regulator that stabilizes basal cytosolic and endoplasmic reticulum Ca2+ levels." Cell **131**(7): 1327-1339.

Cao, Y., G. Bonizzi, et al. (2001). "IKKalpha provides an essential link between RANK signaling and cyclin D1 expression during mammary gland development." Cell **107**(6): 763-775.

Datsenko, K. A. and B. L. Wanner (2000). "One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products." Proc Natl Acad Sci U S A **97**(12): 6640-6645.

Dehio, C., M. C. Prevost, et al. (1995). "Invasion of epithelial cells by Shigella flexneri induces tyrosine phosphorylation of cortactin by a pp60c-src-mediated signalling pathway." Embo J **14**(11): 2471-2482.

Franchi, L., J. H. Park, et al. (2008). "Intracellular NOD-like receptors in innate immunity, infection and disease." Cell Microbiol **10**(1): 1-8.

Galvez, T., M. N. Teruel, et al. (2007). "siRNA screen of the human signaling proteome identifies the PtdIns(3,4,5)P3-mTOR signaling pathway as a primary regulator of transferrin uptake." Genome Biol **8**(7): R142.

Gilmore, T. D. (2006). "Introduction to NF-kappaB: players, pathways, perspectives." Oncogene **25**(51): 6680-6684.

Girardin, S. E., I. G. Boneca, et al. (2003). "Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan." Science **300**(5625): 1584-1587.

Girardin, S. E., R. Tournebize, et al. (2001). "CARD4/Nod1 mediates NF-kappaB and JNK activation by invasive Shigella flexneri." EMBO Rep **2**(8): 736-742.

Hayden, M. S. and S. Ghosh (2004). "Signaling to NF-kappaB." Genes Dev **18**(18): 2195-2224.

Hayden, M. S. and S. Ghosh (2008). "Shared principles in NF-kappaB signaling." Cell **132**(3): 344-362.

Inohara, N., T. Koseki, et al. (2000). "An induced proximity model for NF-kappa B activation in the Nod1/RICK and RIP signaling pathways." J Biol Chem **275**(36): 27823-27831.

Islam, D., B. Veress, et al. (1997). "In situ characterization of inflammatory responses in the rectal mucosae of patients with shigellosis." Infect Immun **65**(2): 739-749.

Izmailova, E. S., N. Paz, et al. (2007). "Use of molecular imaging to quantify response to IKK-2 inhibitor treatment in murine arthritis." Arthritis Rheum **56**(1): 117-128.

Kawai, T. and S. Akira (2008). "Toll-like receptor and RIG-I-like receptor signaling." Ann N Y Acad Sci **1143**: 1-20.

Le Bourhis, L., S. Benko, et al. (2007). "Nod1 and Nod2 in innate immunity and human inflammatory disorders." Biochem Soc Trans **35**(Pt 6): 1479-1484.

Li, H., H. Xu, et al. (2007). "The phosphothreonine lyase activity of a bacterial type III effector family." Science **315**(5814): 1000-1003.

Liou, J., M. L. Kim, et al. (2005). "STIM is a Ca2+ sensor essential for Ca2+-store-depletion-triggered Ca2+ influx." Curr Biol **15**(13): 1235-1241.

Medzhitov, R. (2007). "Recognition of microorganisms and activation of the immune response." Nature **449**(7164): 819-826.

Murphy, K. C. and K. G. Campellone (2003). "Lambda Red-mediated recombinogenic engineering of enterohemorrhagic and enteropathogenic E. coli." BMC Mol Biol **4**: 11.

Ogawa, H., M. Azuma, et al. "IkappaB kinase beta inhibitor IMD-0354 suppresses airway remodelling in a Dermatophagoides pteronyssinus-sensitized mouse model of chronic asthma." Clin Exp Allergy.

Ogawa, M., Y. Handa, et al. (2008). "The versatility of Shigella effectors." Nat Rev Microbiol **6**(1): 11-16.

Pedron, T., C. Thibault, et al. (2003). "The invasive phenotype of Shigella flexneri directs a distinct gene expression pattern in the human intestinal epithelial cell line Caco-2." J Biol Chem **278**(36): 33878-33886.

Pelkmans, L., E. Fava, et al. (2005). "Genome-wide analysis of human kinases in clathrin- and caveolae/raft-mediated endocytosis." Nature **436**(7047): 78-86.

Rasmussen, S. B., L. S. Reinert, et al. (2009). "Innate recognition of intracellular pathogens: detection and activation of the first line of defense." Apmis **117**(5-6): 323-337.

Reijmerink, N. E., R. W. Bottema, et al. "TLR-related pathway analysis: novel gene-gene interactions in the development of asthma and atopy." Allergy **65**(2): 199-207.

Sansonetti, P. J., J. Arondel, et al. (1999). "Interleukin-8 controls bacterial transepithelial translocation at the cost of epithelial destruction in experimental shigellosis." Infect Immun **67**(3): 1471-1480.

Schroeder, G. N. and H. Hilbi (2008). "Molecular pathogenesis of Shigella spp.: controlling host cell signaling, invasion, and death by type III secretion." Clin Microbiol Rev **21**(1): 134-156.

Solt, L. A., L. A. Madge, et al. (2007). "Interleukin-1-induced NF-kappaB activation is NEMO-dependent but does not require IKKbeta." J Biol Chem **282**(12): 8724-8733.

Solt, L. A. and M. J. May (2008). "The IkappaB kinase complex: master regulator of NF-kappaB signaling." Immunol Res **42**(1-3): 3-18.

Takeuchi, O. and S. Akira "Pattern recognition receptors and inflammation." Cell **140**(6): 805-820.

Weidinger, S., N. Klopp, et al. (2005). "Association of NOD1 polymorphisms with atopic eczema and related phenotypes." J Allergy Clin Immunol **116**(1): 177-184.

Zhang, E. E., A. C. Liu, et al. (2009). "A genome-wide RNAi screen for modifiers of the circadian clock in human cells." Cell **139**(1): 199-210.

Ziegelbauer, K., F. Gantner, et al. (2005). "A selective novel low-molecular-weight inhibitor of IkappaB kinase-beta (IKK-beta) prevents pulmonary inflammation and shows broad anti-inflammatory activity." Br J Pharmacol **145**(2): 178-192.

CHAPTER 3

Endocytosis-independent function of clathrin heavy chain in the control of basal NF-κB activation

(MANUSCRIPT SUBMITTED)

Endocytosis-independent function of clathrin heavy chain in the control of basal NF-κB activation

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3.1 ABSTRACT

Background: Nuclear factor-κB (NF-κB) is a transcription factor that regulates the transcription of genes involved in a variety of biological processes, including innate and adaptive immunity, stress responses and cell proliferation. Constitutive or excessive NF-κB activity has been associated with inflammatory disorders and higher risk of cancer. In contrast to the mechanisms controlling inducible activation, the regulation of basal NF-κB activation is not well understood. Here we test whether clathrin heavy chain (CHC) contributes to the regulation of basal NF-κB activity in epithelial cells.

Methodology: Using RNA interference to reduce endogenous CHC expression, we found that CHC is required to prevent constitutive activation of NF-κB and gene expression. Immunofluorescence staining showed constitutive nuclear localization of the NF-κB subunit p65 in absence of stimulation after CHC knockdown. Elevated basal p65 nuclear localization is caused by constitutive phosphorylation and degradation of inhibitor of NF-κB (IκBα) through an IκBα kinase α (IKKα)-dependent mechanism. The role of CHC in NF-κB signaling is functionally relevant as constitutive expression of the proinflammatory chemokine interleukin-8 (IL-8), whose expression is regulated by NF-κB, was found after CHC knockdown. Disruption of clathrin-mediated endocytosis by depletion of the μ2-subunit of the endocytosis adaptor protein AP-2 and knockdown of clathrin light chain a (CHLa), failed to induce constitutive NF-κB activation and IL-8 expression, showing that CHC acts on NF-κB independently of endocytosis and CLCa.

Conclusions: We conclude that CHC functions as a built-in molecular brake that ensures a tight control of basal NF-κB activation and gene expression in unstimulated cells. Furthermore, our data suggest a potential link between a defect in CHC expression and chronic inflammation disorder and cancer.

3.2 INTRODUCTION

Nuclear factor-kappa B (NF-κB) transcription factors control the expression of genes involved in a large spectrum of biological processes, including inflammation, adaptive immunity, stress responses, angiogenesis, cell proliferation and invasion (Basseres and Baldwin 2006; Hayden, West et al. 2006). Aberrant regulation of NF-κB activity has been associated with immune disorders and numerous cancers (Prasad, Ravindran et al.). Although NF-κB has been the subject of intensive investigation, the molecular mechanisms underlying its regulation are not fully understood. There are five NF-κB isoforms in mammalian cells: p65/RelA, RelB, c-Rel, p50 (NF-κB1) and p52 (NF-κB2). All these proteins share a Rel homology domain responsible for homo- and heterodimerization as well as for sequence-specific DNA binding. Among the various hetero-and homodimers formed by NF-κB proteins, the p50/p65 heterodimer is predominant in many cell types (Baldwin 1996). Dimers of NF-κB proteins bind κB sites in promoters or enhancers of target genes and regulate transcription via the recruitment of transcriptional co-activators and co-repressors. A number of posttranslational modifications of the NF-κB proteins, including phosphorylations and acetylations, further modulate DNA binding and, therefore, transcriptional activity (Perkins 2006). In absence of stimulation, most of the NF-κB dimers are retained in the cytoplasm by the Inhibitor of NF-κB (IκB) family members whose prototype is the protein IκBα (Baeuerle and Baltimore 1988; Beg and Baldwin 1993; Baldwin 1996). IκBα contains several ankyrin repeats that mediate the binding to NF-κB dimers and mask the nuclear localization signal (NLS) of p65. Following cell stimulation by proinflammatory cytokines, such as tumor necrosis factor α (TNFα) and

interleukin-1, IκBα is rapidly phosphorylated on serine 32 and serine 36 residues by the IκB kinase (IKK) complex composed of three subunits: two catalytic subunits, IKKα and IKKβ, and the regulatory scaffold component NF-κB essential modulator (NEMO). IκBα phosphorylation is then followed by rapid polyubiquitination and degradation via the 26S proteasome. Released NF-κB dimers translocate into the nucleus where they drive gene expression (Gilmore 2006; Hayden and Ghosh 2008). As the gene encoding IκBα is rapidly upregulated following NF-κB activation, IκBα is promptly resynthesized (Hoffmann, Levchenko et al. 2002). Newly synthesized IκBα proteins bind to nuclear NF-κB dimers and dissociate them from DNA. This mechanism terminates the transcriptional activity of NF-κB and resets gene expression to basal level.

Although constitutive NF-κB activation has been associated with inflammatory disorders and numerous cancers (Prasad, Ravindran et al. ; Yamaguchi, Ito et al. 2009), the mechanisms leading to elevated basal NF-κB activation remain unclear. Proposed mechanisms include activation of kinases, overexpression of cytokines, dysregulation of cell surface receptors and activation of oncoproteins. We recently performed an RNA interference (RNAi) screen targeting host signaling proteins that could potentially be involved in the inflammatory response following infection by *Shigella flexneri* (Kim et al., submitted). From that screen, we identified clathrin heavy chain (CHC) as one of the proteins that, when knocked down, strongly enhanced activation of NF-κB. In this study, we examine the role of CHC in the control of basal NF-κB activation.

CHC is mainly known as a structural component of clathrin and for its role in clathrin-mediated endocytosis (CME) (Royle 2006; Miaczynska and Stenmark 2008). The association of three CHCs and up to three clathrin light chains (CLCs) forms a clathrin

triskelion structure that self-polymerizes to form a curved lattice around invaginated pits. Through this mechanism, CHC is involved in the uptake of nutrients, the internalization of pathogens, the downregulation of certain ligand-induced receptors and in protein sorting at the trans-Golgi network (TGN) during protein secretion (Conner and Schmid 2003; Veiga and Cossart 2005; Miaczynska and Stenmark 2008). However, similar to other endocytic proteins (Pilecka, Banach-Orlowska et al. 2007), CHC appears to perform multiple functions in cells. It has been reported that CHC is involved in chromosome segregation during mitosis (Royle, Bright et al. 2005). In addition, a fraction of CHC proteins that localize to the nucleus bind to the p53-responsive promoter and favor p53-mediated transcription (Enari, Ohmori et al. 2006).

Here we have used RNAi to effectively knock down CHC in epithelial cells. Surprisingly, we found that the depletion of CHC induces constitutive nuclear localization of the NF-κB subunit p65 in absence of stimulation. Elevated basal p65 nuclear localization was associated with constitutive phosphorylation and degradation of IκBα via an IKKα dependent mechanism and constitutive expression of the proinflammatory chemokine interleukin-8 (IL-8), whose expression is regulated by NF-κB. Interestingly, CHC acted on NF-κB independently from its roles in CME and from CLCs. Taken together, our data reveal a new function of CHC in the control of basal NF-κB activity and gene expression in epithelial cells.

3.3 MATERIALS AND METHODS

Antibodies and reagents

Antibodies against NF-κB p65, IκBα, CLCa and IKKα were obtained from Santa Cruz Biotechnology (Santa Cruz, USA) while the anti-CHC antibody was from BD Transduction Laboratories (San Jose, USA). The anti-actin antibody was from Chemicon (Billerica, USA) and the anti-phospho-IκBα was from Cell signaling technology (Beverly, USA). The anti-mouse IgG-Cy5 was obtained from Zymed (San Francisco, USA) and the anti-rabbit IgG-HRP and anti-mouse IgG-HRP from GE Healthcare (Pittsburgh, USA). Hoeschst and FITC-phalloidin were from Invitrogen (Carlsbad, USA).

Cell culture and siRNA transfection

HeLa Kyoto (Neumann, Held et al. 2006) and MCF-7 cells (ATCC, Manassas, USA) were maintained in Dulbecco's modified Eagle's medium (high glucose) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in 10% CO_2. HeLa and MCF-7 cells were transfected with different siRNAs at 10 nM using Lipofectamine 2000 (Invitrogen, Carlsbad, USA). ON-TARGETplus SMARTpool siRNAs for clathrin heavy chain (CHC/CLTC, #L-004001-00-005), clathrin light chain a (CLCa/CLTA, #L-004002-00-005), AP2M1 (#L-008170-00-005), IKKα (#L-003473-00-005) and ON-TARGETplus siCONTROL were obtained from Dharmacon (Dallas, USA).

Immunofluorescence and microscopy

Cells were fixed with 4% PFA for 6 min and permeabilized in 0.5% Triton X-100 for 10 min. They were, then, incubated with a mouse monoclonal anti-p65 antibody (1 μg/ml) overnight at 4 °C and stained with a Cy5-conjugated secondary antibody and Hoechst (10 μg/ml) for 40 min at room temperature. Images were acquired at 12 random sites of each well using the automated ImageXpress microscope (Molecular devices, Sunnyvale, USA). The nuclear localization of p65 was automatically quantified by using the Enhanced-Translocation module of MetaXpress (Molecular devices, Sunnyvale, USA). Briefly, the Hoechst staining was used as a mask to automatically identify nuclei in the p65 staining image. The cytoplasmic area of each cell was defined by a ring around the nucleus. For each cell, the ratio of p65 intensity in the nucleus and in the cytoplasmic ring defined as the Nuc/Cyt p65 NF-κB ratio was calculated and averaged over several thousands of cells per well.

Transferrin uptake assay

Transferrin uptake was measured as described by Galvez T. et al (Galvez, Teruel et al. 2007). Briefly, HeLa cells were treated with Alexa 594-conjugated transferrin (Invitrogen) for 10 min followed by a quick acid wash to cleave off the receptor-bound transferrin from the plasma membrane. Cells were then fixed with 4% PFA and stained with Hoechst. Transferrin uptake was automatically quantified by using the Multi-wave Length Cell Scoring module of MetaXpress (Molecular devices, Sunnyvale, USA).

Enzyme- linked Immunosorbent Assay (ELISA)

IL-8 secretion was measured by ELISA in the supernatant of siRNA-transfected HeLa and MCF-7 cells, 72 hours post transfection. Cell-free supernatants from triplicate wells were analyzed for their IL-8 content using a commercial ELISA kit (BD Pharmingen, San Jose, USA). In parallel, cells from the plate were stained with Hoechst to quantify cell numbers. IL-8 measurements were normalized to the number of cells for each condition.

Western Blot Analysis

HeLa or MCF-7 cells were transfected with siRNAs in a 6-well plate. 72 hours post transfection, cells were lysed in Phosphosafe Extraction Buffer (Novagen, Darmstadt, Germany) supplemented with 1x protease inhibitor cocktail (Calbiochem, Darmstadt, Germany). Protein concentration was measured using the bicinchoninic acid (BCA) kit (Pierce, Rockford, USA). Equal amounts of proteins were resolved by SDS-PAGE and transferred to Hybond C-Extra membrane (Amersham Bioscience, Pittsburgh, USA) for immunoblotting with indicated antibodies. Primary antibodies were detected using horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibodies, and visualized with the ECL system (Pierce). Quantification of the blots was performed using the densitometry feature of Photoshop.

Quantitative real-time PCR

Total RNA was isolated from control or CHC siRNA transfected cells in a 6-well plate using the total RNA purification system (Invitrogen, Carlsbad, USA). cDNAs were generated by using the Superscript III 1st Strand Synthesis Kit (Invitrogen, Carlsbad, USA). Real-time PCR was performed on an ABI Prism 7700 system (Applied Biosystems,

Foster city, USA) using the SYBR green PCR Master Mix (Applied Biosystems, Foster city, USA) to measure relative IκBα mRNA level in CHC-depleted and control cells. GAPDH was used as an internal control to normalize mRNA expression. Each sample was analyzed in triplicate. The primer sequences used are as follows. IκBα-forward: 5'-GACCTGGTGTCACTCCTGTTG; IκBα-reverse: 5'-CTCTCCTCATCCTCACTCTCTGG; GAPDH-forward: 5'-GAAGGTGAAGGTCG GAGTC; GAPDH-reverse: 5'-GAAGATGGTGATGGGATTTC.

Statistical analysis

Results are expressed as the mean ± SD as specified in figure legends. p values were calculated with a two-tailed two-sample equal variance t-test. p values of less than 0.05 were considered statistically significant.

3.4 RESULTS

3.4.1 CHC prevents constitutive NF-κB p65 nuclear localization in unstimulated epithelial cells

To investigate the implication of CHC in the regulation of basal NF-κB activation, we tested whether CHC interfered with the localization of the NF-κB subunit p65 in absence of stimulation. For this purpose, HeLa cells were depleted of CHC by transfection with a pool of four siRNAs targeting CHC. A pool of four none-targeting siRNAs was used as control in parallel. The efficiency of knockdown after 72 hours was controlled by measuring the expression of CHC by western immunoblotting (Figure 3.1A). The localization of p65 was visualized by immunofluorescence microscopy with an anti-p65 antibody. As expected, p65 was mostly present in the cytoplasm of control cells (Figure 3.1B, left panel). Surprisingly, both cytoplasmic and nuclear localization of p65 was observed after CHC knockdown (Figure 3.1B, right panel). This observation was confirmed by quantification with automated image processing of the nuclear/cytoplasmic p65 intensity ratio (Figure 3.1C). These results showed that CHC expression is required to prevent constitutive nuclear localization of p65 in unstimulated HeLa cells.

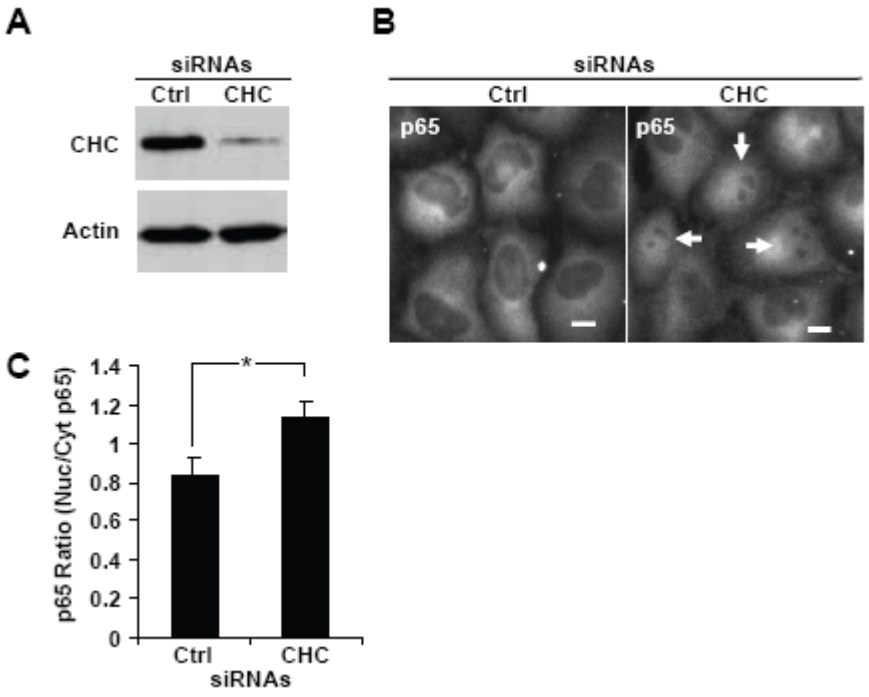

Figure 3.1 CHC prevents constitutive NF-κB p65 nuclear localization in unstimulated epithelial cells.

(**A**) Effective knockdown of CHC after siRNA transfection. Lysates from HeLa cells transfected with control (Ctrl) or CHC siRNAs for 72 hours were analyzed by western immunoblotting using the indicated antibodies. Actin is shown as a loading control. (**B**) Constitutive nuclear localization of p65 after CHC knockdown. HeLa cells were transfected with either control or CHC siRNA and p65 localization was visualized by immunofluorescence microscopy. White arrows indicate cells showing a clear nuclear localization of p65. Scale bars, 10 μm. (**C**) Quantification of the nuclear/cytosolic p65 intensity ratio in control and CHC siRNA transfected HeLa cells (results are expressed as the mean ± SD of 12 images; *p=3.14E-07, graph representative of 3 independent experiments).

3.4.2 CHC prevents constitutive IKK-mediated phosphorylation and degradation of IκBα in unstimulated epithelial cells

The localization of p65 results from a complex equilibrium between cytoplasm to nucleus translocation and nuclear export (Hoffmann, Levchenko et al. 2002). We analyzed, most specifically, the implication of CHC in the mechanisms that control the nuclear translocation of p65. In the canonical NF-κB activation pathway triggered by most stimuli, this process is tightly controlled by IKK complex-dependent phosphorylation and proteolytic degradation of IκB proteins. As we observed more p65 in the nuclei of CHC-depleted cells, we hypothesized that basal IκB degradation was elevated in these cells. To directly test this assumption, the level of IκBα in CHC and control siRNA transfected cells was analyzed by western immunoblotting. As shown in Figure 3.2A, a strong reduction in the level of IκBα was found after CHC knockdown. To exclude the hypothesis that this diminution resulted from reduced *IκBα* gene transcription, the level of IκBα mRNA was analyzed by quantitative real-time PCR. A two-fold increase in IκBα mRNA was measured after knockdown compared to control (Figure 3.2B), indicating that the reduction of IκBα level was not caused by an inhibition of transcription but, most likely, by constitutive degradation of IκBα proteins. Because the catalytic subunit IKKα largely contributes to IκBα phosphorylation and degradation in HeLa cells (Adli, Merkhofer et al.), we examined the effect of IKKα knockdown on constitutive IκBα degradation. For this purpose, HeLa cells were transfected with combinations of CHC and IKKα siRNAs for single or co-depletion experiments as described in Figure 3.2C. When IKKα was depleted, the knockdown of CHC had no effect on the level of IκBα (Figure 3.2C), showing that CHC

controls basal IκBα degradation by a mechanism dependent on IKKα. IκBα proteins are subject to phosphorylation by the IKK complex prior to degradation. Therefore we tested if constitutive IκBα degradation resulted from an increase of IκBα phosphorylation (p-IκBα). The phosphorylation at position serine 32 was analyzed by western immunoblotting using a phospho-specific antibody. Interestingly, a two-fold increase in the level of p-IκBα was observed in CHC-depleted cells (Figures 3.2D and 3.2E) after normalization to the level of IκBα, suggesting that constitutive degradation of IκBα was caused by constitutive IκBα phosphorylation. Taken together, these results showed that CHC prevents constitutive NF-κB activation in unstimulated HeLa cells by blocking the spontaneous phosphorylation and degradation of IκBα by the IKK complex.

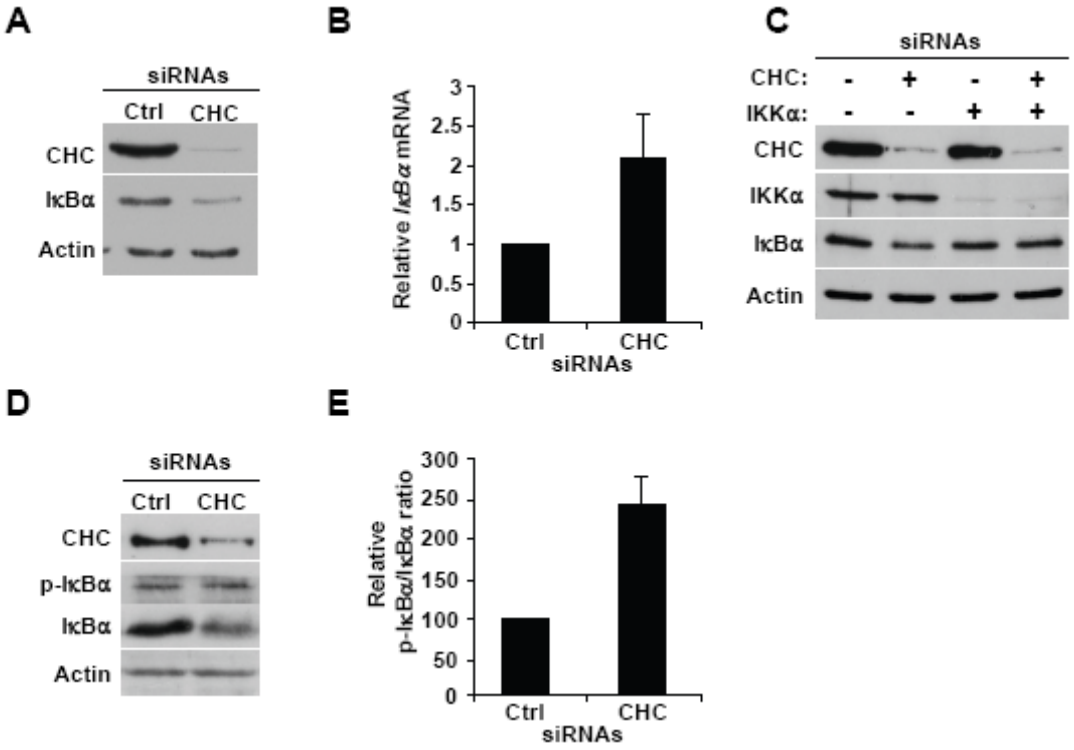

Figure 3.2 CHC prevents constitutive degradation and phosphorylation of IκBα by an IKKα-dependent mechanism.

(**A**) Reduced level of IκBα after CHC knockdown in HeLa cells. Cell lysates from control or CHC siRNA transfected cells were analyzed by western immunoblotting using the indicated antibodies. Actin is shown as a loading control (representative of 3 independent experiments). (**B**) Quantification of the level of *IκBα* mRNA by quantitative RT-PCR in control or CHC-depleted HeLa cells. *GAPDH* mRNA was used as an internal control for normalization (results are expressed as the mean ± SD of 3 independent experiments). (**C**) IKKα-depletion abolishes the constitutive degradation of IκBα induced by CHC knockdown. Lysates from cells transfected with different combinations of IKKα and CHC siRNAs were analyzed by immunoblotting using the indicated antibodies. Total siRNA concentration was kept constant by adding appropriate amounts of control siRNAs. Actin is shown as a loading control (data representative of 2 independent experiments). (**D**) CHC prevents enhanced basal phosphorylation of IκBα at position serine 32. Lysates from control or CHC-depleted HeLa cells were analyzed by immunoblotting using the indicated antibodies. Actin is shown as a loading control. (**E**) Densitometric quantification of the p-IκBα/IκBα ratio (results are expressed as the mean ± SD of 3 independent experiments).

3.4.3 CHC prevents constitutive IL-8 secretion in unstimulated epithelial cells

Previous results indicated that CHC was required to prevent constitutive p65 nuclear translocation. Because this process directly contributes to the regulation of gene expression, we tested whether the presence of CHC was also necessary to prevent constitutive expression of genes regulated by NF-κB. In particular, we investigated the expression of the proinflammatory chemokine IL-8. IL-8 secretion was measured by ELISA in the supernatant of CHC and control siRNA transfected HeLa cells. In line with the results obtained on NF-κB activation, knocking down CHC strongly enhanced basal IL-8 secretion (Figure 3.3A), showing that, indeed, the expression of CHC was critical to prevent constitutive IL-8 expression in HeLa cells. The same result was obtained in the

breast cancer cell line MCF-7 (Figure 3.3B). Furthermore, consistent with the results obtained on IκBα degradation, constitutive expression of IL-8 was massively reduced when IKKα was knocked down (Figure 3.3C). Taken together, these results showed that CHC prevents constitutive expression of IL-8, and that this new function of CHC in NF-κB signaling depends on IKKα and corresponds to a general mechanism taking place in different cells lines.

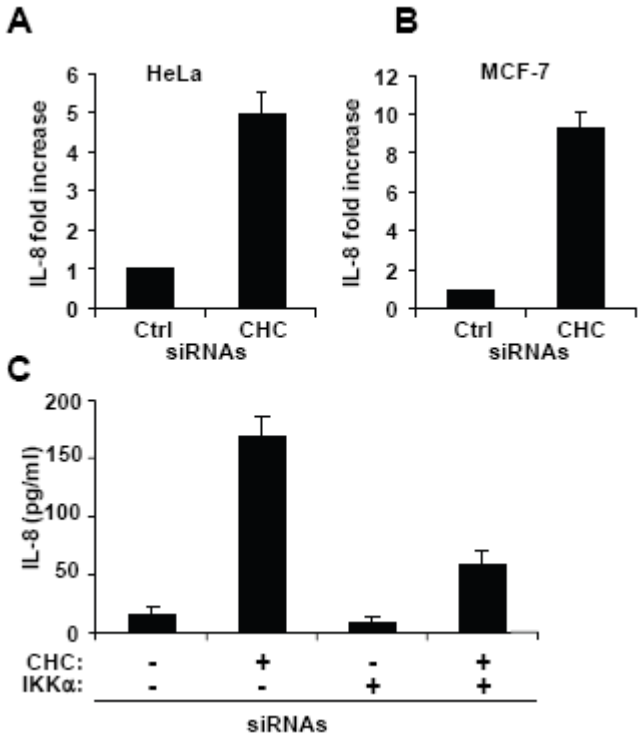

Figure 3.3 CHC prevents constitutive IL-8 expression in unstimulated epithelial cells

(**A**) Constitutive IL-8 expression after knockdown of CHC in HeLa cells. Cells were transfected with control or CHC siRNAs. After 72 hours, supernatants were collected and analyzed for their content in IL-8 by ELISA (results are expressed as the mean ± SD of 3 independent experiments). (**B**) Constitutive IL-8 expression after knockdown of CHC in MCF-7 cells. MCF-7 cells were treated as described in (A) (results are expressed as the mean ± SD of 3 independent experiments). (**C**) IKKα-depletion abolishes the constitutive secretion of IL-8 induced by CHC knockdown. HeLa cells were transfected with different combinations of IKKα and CHC siRNAs for 72 hours. Total siRNA concentration was kept constant by adding appropriate amounts of control siRNAs.

Supernatants were collected to measure the concentration of IL-8 by ELISA (results are expressed as the mean ± SD of 3 independent experiments).

3.4.4 CHC controls basal NF-κB activation independently of endocytosis and clathrin light chains

Through its activity in CME, CHC is involved in the internalization of nutrients, pathogens, antigens, growth factors and receptors (Conner and Schmid 2003; Veiga and Cossart 2005; Miaczynska and Stenmark 2008). To test whether CHC regulated indirectly the NF-κB pathway via its function in CME, we measured p65 nuclear translocation and IL-8 secretion in cells where CME was disrupted by RNAi-mediated depletion of the μ2-subunit of the main CME adaptor protein AP-2 (AP2M1). The recruitment of AP-2 at the plasma membrane is critical for the initiation of CME (Motley, Bright et al. 2003). AP-2 interacts with sorting signals present in the cytoplasmic domains of membrane proteins destined to become cargo in the coated vesicles. In addition, AP-2 recruits clathrin onto the membrane, where it functions as a scaffold for vesicle budding. First, in order to demonstrate that CME was impaired in AP2M1 and CHC-depleted cells, the CME-dependent mechanism of transferrin uptake was monitored in HeLa cells. As previously reported (Motley, Bright et al. 2003), depletion of both CHC and AP2M1 impaired the uptake of fluorescently labeled transferrin (Figures 3.4A and 3.4B). However, although the depletion of AP2M1 blocked transferrin uptake to the same extent as CHC knockdown, it failed to increase basal IκBα degradation (Figures 3.4C and 3.4D) and IL-8 secretion (Figure 3.4E), indicating that CHC controls basal NF-κB activation and gene expression independently of its activity in CME.

CHC is associated to CLCs in clathrin triskelion structures. The functional roles of CLCs have been recently characterized using RNAi. Knocking down CLCs has no effect on CME or the formation of clathrin-coated pits (Poupon, Girard et al. 2008). However, it causes alterations in protein trafficking at the TGN resulting from disruption of huntingtin interacting protein 1 related (HIPR1) recruitment to clathrin-coated structures and disorganization of the actin cytoskeleton (Poupon, Girard et al. 2008). Since CLCs are unstable unless they are bound to CHC (Hinrichsen, Harborth et al. 2003), we tested whether the effect of CHC depletion on NF-κB activation was indirectly due to CLC degradation. For this purpose, IκBα degradation and IL-8 secretion were analyzed in HeLa cells depleted of the protein clathrin light chain a (CLCa) by RNAi. Although the degree of CLCa depletion detected by western immunoblotting was similar in cells transfected with CHC and CLCa siRNAs (Figure 3.4F), knocking down CLCa failed to induce constitutive IκBα degradation (Figures 3.4F and 3.4G) and IL-8 secretion (Figure 3.4H). These results showed that the effects of CHC depletion on NF-κB signaling were directly caused by the depletion of CHC and not by the associated depletion of CLCa. Taken together, these results strongly indicated that CHC prevents constitutive NF-κB activation independently of endocytosis and clathrin light chains.

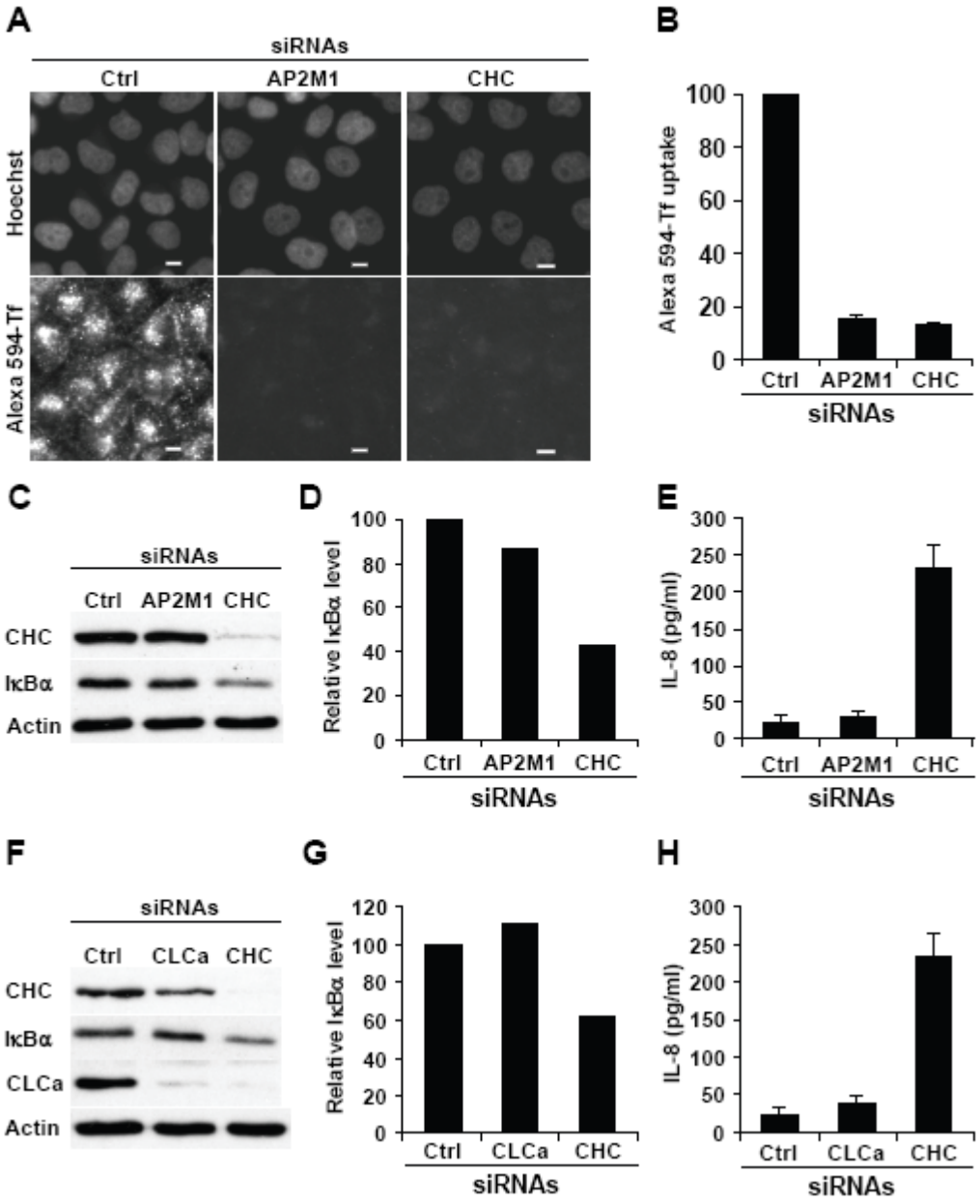

Figure 3.4 CHC regulates NF-κB activation independently of endocytosis and CLCa.

(**A**) Uptake of Alexa 594-transferrin (Alexa 594-Tf) in cells transfected with control (left panels), AP2M1 (middle panels) or CHC (right panels) siRNAs. Scale bars, 10 μm (**B**) Quantification of transferrin uptake by automated image analysis (results are expressed as the mean ± SD of 12 images; graph representative of 2 independent experiments). (**C**) AP2M1 knockdown fails to enhance IκBα degradation. Cell lysates from control, AP2M1 or CHC siRNA-transfected cells were analyzed by immunoblotting using indicated antibodies. Actin is shown as a loading control. (**D**) Densitometric quantification of the levels of IκBα shown in Figure 3.4C (graph representative

of 2 independent experiments). (**E**) AP2M1 knockdown fails to induce constitutive IL-8 expression. HeLa cells were transfected with control, AP2M1 or CHC siRNAs for 72 hours. Supernatants were collected to measure the concentration of IL-8 by ELISA (results are expressed as the mean ± SD of 3 independent experiments). (**F**) CLCa knockdown fails to enhance IκBα degradation. Cell lysates from control, CLCa or CHC siRNA transfected cells were analyzed by immunoblotting using indicated antibodies. Actin is shown as loading control. (**G**) Densitometric quantification of IκBα levels shown in Figure 3.4F (Graph representative of 2 independent experiments). (**H**) CLCa knockdown fails to induce constitutive IL-8 expression. HeLa cells were transfected with control, CLCa or CHC siRNAs for 72 hours and supernatants were collected to measure the concentration of IL-8 by ELISA (results are expressed as the mean ± SD of 3 independent experiments).

3.5 DISCUSSION

Most research groups investigating the regulation of NF-κB activation have focused their studies on the mechanisms induced after cell exposure to various stimuli, including inflammatory cytokines and microbial products. In particular, the canonical pathway of NF-κB activation that depends on the phosphorylation and degradation of IκB proteins downstream of the activation of the IKK complex has been well characterized. In contrast, although constitutive NF-κB activation has been associated with inflammatory disorders and numerous cancers (Prasad, Ravindran et al. ; Yamaguchi, Ito et al. 2009), the mechanisms that lead to elevated basal NF-κB activation remain unclear.

Here we show that CHC functions as a built-in molecular brake that ensures a tight control of basal NF-κB activation and gene expression by preventing constitutive nuclear localization of p65 in absence of stimulation. Using RNAi to reduce cellular levels of CHC, we found that CHC is required for the proper spatial regulation of p65 in unstimulated epithelial cells. Whereas p65 was almost exclusively localized in the cytoplasm of control cells, both cytoplasmic and nuclear localization was observed in cells depleted of CHC. The localization of p65 is largely dependent on IκB proteins that sequestrate the transcription factor in the cytoplasm. Genetic deletion or mutations of IκBα lead to constitutive nuclear localization and NF-κB activation (Beg, Sha et al. 1995; Yamaguchi, Ito et al. 2009). In line with these studies, we found that the effect of CHC depletion on p65 was associated with a strong reduction in the level of IκBα, suggesting that constitutive p65 nuclear localization was likely due to reduced IκBα level. Quantification of IκBα mRNA by quantitative real time PCR revealed that the level of *IκBα* mRNA was slightly

elevated in CHC depleted cells. This result, which can be explained by the fact that *IκBα* is a target gene of NF-κB that is upregulated by constitutive NF-κB activation, indicated that the reduction of IκBα found in CHC depleted cells, was not due to transcriptional inhibition but to elevated basal IκBα degradation. This hypothesis was further supported by data showing that the knockdown of CHC induced constitutive phosphorylation of IκBα at position 32, a phosphorylation event critical to target IκBα for rapid degradation via the ubiquitin proteasome pathway. The role of CHC in the control of basal NF-κB activation was functionally relevant. Indeed a strong induction of IL-8, whose expression is controlled by NF-κB, was observed in cells depleted of CHC showing that, via its activity on NF-κB, CHC participates to gene regulation. Interestingly, a role of CHC in p53-mediated transcription has been recently reported. Enari et al. showed that a fraction of CHC proteins that localize to the nucleus bind to p53-responsive promoters and favor transcription by stabilizing p53 interactions with proteins such as the histone acetyltransferase p300 (Enari, Ohmori et al. 2006). Although CHC contributes to p53 and NF-κB-regulated gene expression by different mechanisms, our data provide a second set of evidence for a role of CHC in gene regulation.

Via its role in CME, CHC is involved in many cellular processes including intracellular trafficking of receptors and nutrient uptake (Miaczynska and Stenmark 2008). To investigate whether CHC indirectly affected basal NF-κB activation via its implication in CME, we analyzed the level of IκBα and IL-8 expression in conditions where CME was disrupted by the depletion of the μ2-subunit of the main CME adaptor AP-2. Whereas the CME-dependent process of transferrin uptake was almost completely abolished, AP2M1 depletion had no effect on the NF-κB signaling pathway, suggesting that CHC was

involved in the NF-κB pathway independently of endocytosis. Although we cannot completely rule out the contribution of a CME mechanism independent of AP-2, these results provide strong evidence for an endocytosis-independent function of CHC in NF-κB-mediated gene regulation. As CLCs are unstable unless bound to CHC, the knockdown of CHC is associated with cellular depletion of CLCs. Interestingly, we showed that the depletion of CLCa has no effect on IκBα degradation and IL-8 expression, indicating that CHC alone contributes to the regulation of the NF-κB pathway. It also suggested that CHC functions in this pathway independently of the roles that CHC and CLCa share in protein trafficking at the TGN (Poupon, Girard et al. 2008).

A raising number of proteins involved at different levels of the signaling pathway contribute to the tight control of basal NF-κB activation in resting cells (Prasad, Ravindran et al.). For instance, expression of the tumor suppressor Gprc5a prevents constitutive p65 nuclear localization and NF-κB activation (Deng, Fujimoto et al.). Although the mechanism remains unclear, the authors propose that the presence of Gprc5a may promote the stabilizing interaction between β-arrestin and IκBα. The role of silencer of death domain (SODD) has also been reported (Jiang, Woronicz et al. 1999). SODD deficiency leads to an increase of NF-κB activation and cytokine expression in absence of stimulation. This protein functions as a gatekeeper that constitutively associates with the cytoplasmic death domain of TNF receptors (TNFR) and blocks TNFR signaling in the absence of ligand. Constitutive activity of NF-κB was also described in c-Abl null fibroblasts (Liberatore, Goff et al. 2009). In contrast to previous mechanisms, unstimulated fibroblasts did not exhibit an increase in IκBα degradation or p65 nuclear translocation but reduced levels of the negative regulator histone deacetylase HDAC1. The mechanism by which

CHC functions in NF-κB signaling remains to be elucidated. Based on our data, we propose that this protein acts as a built-in molecular break that prevents the spontaneous activation of the IKK complex. This hypothesis is supported by the observation that constitutive IκBα degradation and IL-8 secretion were almost completely abolished when IKKα was depleted, and that IκBα was enhanced after CHC knockdown. Because the localization of IKKs is critical for their activity (Weil, Schwamborn et al. 2003), the implication of CHC in the sub-cellular distribution of the IKK complex should be further investigated.

We also report here that CHC impedes constitutive IL-8 secretion by unstimulated HeLa and MCF-7 cells. It is well established that constitutive IL-8 secretion by epithelial cells can lead to chronic recruitment of macrophages that produce and secrete into the microenvironement a variety of cytokines, chemokines and growth factors involved in inflammation-related diseases such as inflammatory bowel disease. In addition, some of these factors promote angiogenesis and act directly on epithelial cells to favor adenoma formation and progression to adenocarcinomas (Basseres and Baldwin 2006). Therefore by showing that CHC prevents constitutive expression of the proinflammatory and tumorigenic factor IL-8, our data suggest that alterations in CHC expression may be associated with chronic inflammation disorder or cancer. As a consequence, CHC expression, that can be regulated by external stimuli such as androgens (Prescott and Tindall 1998), should be systematically investigated in tumors and inflamed tissues.

3.6 ACKNOWLEDGMENTS

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3.7 REFERENCES

Adli, M., E. Merkhofer, et al. "IKKalpha and IKKbeta each function to regulate NF-kappaB activation in the TNF-induced/canonical pathway." PLoS One **5**(2): e9428.

Baeuerle, P. A. and D. Baltimore (1988). "I kappa B: a specific inhibitor of the NF-kappa B transcription factor." Science **242**(4878): 540-546.

Baldwin, A. S., Jr. (1996). "The NF-kappa B and I kappa B proteins: new discoveries and insights." Annu Rev Immunol **14**: 649-683.

Basseres, D. S. and A. S. Baldwin (2006). "Nuclear factor-kappaB and inhibitor of kappaB kinase pathways in oncogenic initiation and progression." Oncogene **25**(51): 6817-6830.

Beg, A. A. and A. S. Baldwin, Jr. (1993). "The I kappa B proteins: multifunctional regulators of Rel/NF-kappa B transcription factors." Genes Dev **7**(11): 2064-2070.

Beg, A. A., W. C. Sha, et al. (1995). "Constitutive NF-kappa B activation, enhanced granulopoiesis, and neonatal lethality in I kappa B alpha-deficient mice." Genes Dev **9**(22): 2736-2746.

Conner, S. D. and S. L. Schmid (2003). "Regulated portals of entry into the cell." Nature **422**(6927): 37-44.

Deng, J., J. Fujimoto, et al. "Knockout of the tumor suppressor gene Gprc5a in mice leads to NF-kappaB activation in airway epithelium and promotes lung inflammation and tumorigenesis." Cancer Prev Res (Phila Pa) **3**(4): 424-437.

Enari, M., K. Ohmori, et al. (2006). "Requirement of clathrin heavy chain for p53-mediated transcription." Genes Dev **20**(9): 1087-1099.

Galvez, T., M. N. Teruel, et al. (2007). "siRNA screen of the human signaling proteome identifies the PtdIns(3,4,5)P3-mTOR signaling pathway as a primary regulator of transferrin uptake." Genome Biol **8**(7): R142.

Gilmore, T. D. (2006). "Introduction to NF-kappaB: players, pathways, perspectives." Oncogene **25**(51): 6680-6684.

Hayden, M. S. and S. Ghosh (2008). "Shared principles in NF-kappaB signaling." Cell **132**(3): 344-362.

Hayden, M. S., A. P. West, et al. (2006). "SnapShot: NF-kappaB signaling pathways." Cell **127**(6): 1286-1287.

Hinrichsen, L., J. Harborth, et al. (2003). "Effect of clathrin heavy chain- and alpha-adaptin-specific small inhibitory RNAs on endocytic accessory proteins and receptor trafficking in HeLa cells." J Biol Chem **278**(46): 45160-45170.

Hoffmann, A., A. Levchenko, et al. (2002). "The IkappaB-NF-kappaB signaling module: temporal control and selective gene activation." Science **298**(5596): 1241-1245.

Jiang, Y., J. D. Woronicz, et al. (1999). "Prevention of constitutive TNF receptor 1 signaling by silencer of death domains." Science **283**(5401): 543-546.

Liberatore, R. A., S. P. Goff, et al. (2009). "NF-kappaB activity is constitutively elevated in c-Abl null fibroblasts." Proc Natl Acad Sci U S A **106**(42): 17823-17828.

Miaczynska, M. and H. Stenmark (2008). "Mechanisms and functions of endocytosis." J Cell Biol **180**(1): 7-11.

Motley, A., N. A. Bright, et al. (2003). "Clathrin-mediated endocytosis in AP-2-depleted cells." J Cell Biol **162**(5): 909-918.

Neumann, B., M. Held, et al. (2006). "High-throughput RNAi screening by time-lapse imaging of live human cells." Nat Meth **3**(5): 385-390.

Perkins, N. D. (2006). "Post-translational modifications regulating the activity and function of the nuclear factor kappa B pathway." Oncogene **25**(51): 6717-6730.

Pilecka, I., M. Banach-Orlowska, et al. (2007). "Nuclear functions of endocytic proteins." Eur J Cell Biol **86**(9): 533-547.

Poupon, V., M. Girard, et al. (2008). "Clathrin light chains function in mannose phosphate receptor trafficking via regulation of actin assembly." Proc Natl Acad Sci U S A **105**(1): 168-173.

Prasad, S., J. Ravindran, et al. "NF-kappaB and cancer: how intimate is this relationship." Mol Cell Biochem **336**(1-2): 25-37.

Prescott, J. L. and D. J. Tindall (1998). "Clathrin gene expression is androgen regulated in the prostate." Endocrinology **139**(4): 2111-2119.

Royle, S. J. (2006). "The cellular functions of clathrin." Cell Mol Life Sci **63**(16): 1823-1832.

Royle, S. J., N. A. Bright, et al. (2005). "Clathrin is required for the function of the mitotic spindle." Nature **434**(7037): 1152-1157.

Veiga, E. and P. Cossart (2005). "Listeria hijacks the clathrin-dependent endocytic machinery to invade mammalian cells." Nat Cell Biol **7**(9): 894-900.

Weil, R., K. Schwamborn, et al. (2003). "Induction of the NF-kappaB cascade by recruitment of the scaffold molecule NEMO to the T cell receptor." Immunity **18**(1): 13-26.

Yamaguchi, N., T. Ito, et al. (2009). "Constitutive activation of nuclear factor-kappaB is preferentially involved in the proliferation of basal-like subtype breast cancer cell lines." Cancer Sci **100**(9): 1668-1674.

CONCLUSIONS AND OUTLOOK

Role of IKKα in inflammation and innate immunity

Pro-inflammatory cytokines such as TNFα and IL-1, and many pathogens including *S. flexneri* activate the transcription factor NF-κB via the so-called canonical pathways, which results in the expression of chemokines such as IL-8, one of the key mediators in inflammation and innate immunity (Akira, Uematsu et al. 2006; Lawrence 2009). Ample evidence indicates that in the canonical NF-κB pathways IKKβ subunit of the IKK complex is essential for phosphorylation and degradation of IκBα, causing it to dissociate from NF-κB and allowing NF-κB to translocate to the nucleus, whereas IKKα subunit regulates the non-canonical or alternative pathway important for B-cell maturation and lymphoid organogenesis rather than inflammation and innate immunity (Karin and Ben-Neriah 2000; Bonizzi and Karin 2004; Dejardin 2006; Lawrence and Bebien 2007). In contrast to this dominant model of IKKβ-dependent NF-κB activation in the canonical pathways, we have shown here for the first time that IKKα also regulates the canonical NF-κB activation and subsequent IL-8 expression downstream of Nod1-mediated peptidoglycan recognition during *S. flexneri* infection of HeLa cells. A very recent study also shows that both IKKα and IKKβ contribute to IκBα phosphorylation and NF-κB activation in response to TNFα stimulation in HeLa cells (Adli, Merkhofer et al. 2010), supporting the role of IKKα in the canonical NF-κB activation. Therefore, it is conceivable that IKKα as well as IKKβ plays an essential role in inflammation and innate immunity. However, it still remains an open question as to how IKKα and IKKβ stay as an inactive complex in resting cells and gets rapidly activated upon stimulation.

Mechanism of IKKα activation

A putative model for the activation of IKKα and IKKβ is their activation loop (T-loop) phosphorylation in the N-terminal kinase domain by upstream kinases such as TAK1 (Hayden and Ghosh 2008). Thus, mutation of the serine residues (S176/S180 for IKKα and S177/S181 for IKKβ) in the T-loop to alanines abrogates NF-κB activation in response to TNFα stimulation (Hacker and Karin 2006). However, different from the TNFR signaling, phosphorylation at the two serine residues was barely detectable in response to *S.flexneri* infection or Tri-DAP treatment up to 60 minutes when IκBα degradation occurs maximally (unpublished data), suggesting that Nod1-mediated NF-κB activation might be independent of the T-loop phosphorylation of IKKα and IKKβ. This may further indicate that the serine residues in the T-loop of IKKα and IKKβ may be structurally required for NF-κB activation but the serines phosphorylation may not be functionally responsible for IκBα phosphorylation since IκBα phosphorylation and degradation occurs in the absence of IKK phosphorylation upon *S.flexneri* infection or Nod1 stimulation. A study showed that in addition to phosphorylation of two serine residues in the T-loop, phosphorylation of two tyrosine residues (Y187/Y198 for IKKα and Y188/Y199 for IKKβ) is induced by protein kinase C-dependent c-Src activation and is important for TNFα-induced NF-κB activation and subsequent target gene expression (Huang, Chen et al. 2003; Huang, Chen et al. 2003). Tyrosine phosphorylation of IKKα and IKKβ in the Nod1 signaling pathways is yet unknown. In an independent study, we observed that *S.flexneri* infection or Tri-DAP treatment of HeLa cells induced strong tyrosine phosphorylation of multiple undefined proteins, which is detected by both immnofluorescence and western blot analysis using an anti-phospho tyrosine (pY) antibody that specifically binds to tyrosine phosphorylated proteins (unpublished data). Given that Src is a tyrosine kinase known to be activated during *Shigella* entry process (Dehio, Prevost et al. 1995), it would be worth

investigating whether or not Src can induce tyrosine phosphorylation of IKKα and IKKβ during *S.flexneri* infection, and tyrosine phosphorylation of IKKα and IKKβ are responsible for NF-κB activation.

IKKα and histone phosphorylation

In addition to its activity toward IκBα phosphorylation, IKKα is involved in histone H3 phosphorylation, which facilitates NF-κB binding to IL-8 promoter (Yamamoto, Verma et al. 2003). Previous studies showed that IL-8 is poorly secreted during *S.flexneri* infection in part due to the downregulation of host signalling by the *Shigella* type-III effectors (Arbibe, Kim et al. 2007; Zurawski, Mumy et al. 2009). Especially, the nucleus-targeted OspF dephosphorylates p38 and ERK, which are responsible for histone H3 Ser10 phosphorylation (Arbibe, Kim et al. 2007; Li, Xu et al. 2007). Interestingly, I have recently found that IKKα and IKKβ phosphorylation at the two serine residues in the T-loop is induced not by wild type *S.flexneri* but by the *OspF*-deficient one (unpublished data). It will be of particular interest to study first of all, the previously unknown implication of S176/S180 phosphorylation of IKKα in the regulation of histone phosphorylation rather than IκBα phosphorylation and secondly, the unknown but possible connection between IKK and p38 (and ERK) as both are involved in histone H3 phosphorylation.

In conclusion, this part of my thesis on IKKα not only shows the previously unknown function of IKKα in inflammation and innate immunity during bacterial infection but also may draw further attention for the IKKα-dependent NF-κB activation in many other canonical pathways. Furthermore, this finding may present new therapeutic opportunities for IKKα inhibitors in the treatment of infectious diseases.

Inhibition of basal NF-κB activation by clathrin heavy chain

Constitutive NF-κB activation has been associated with many chronic inflammatory diseases such as cancer (Karin and Greten 2005), but the underlying molecular mechanism by which basal NF-κB is elevated remains unclear. I showed in the second part of my dissertation that depletion of clathrin heavy chain (CHC) resulted in constitutive IκBα degradation, NF-κB activation and high level of IL-8 secretion in resting cells. Normally, NF-κB is retained in the cytoplasm of resting cells through an interaction with IκBα, thereby preventing undesired activation of NF-κB (Liou and Baltimore 1993; Hayden and Ghosh 2008). Thus, genetic deletion of *IκBα* leads to constitutive NF-κB activation (Beg, Sha et al. 1995). However, unlike the constitutive NF-κB activation by genetic deletion of *IκBα*, which is simply due to the absence of IκBα expression that can bind to and retain NF-κB in the cytoplasm, CHC depletion induced higher mRNA expression of *IκBα*, indicating a constant turnover of IκBα at the post-translational level through an active event upstream of IκBα such as an activation of IKK complex.

Inhibition of basal IKK activation and IκBα turnover by clathrin heavy chain

Degradation of IκBα is induced by cytokines such as TNFα and bacterial products such Tri-DAP and is mediated by IKKα- and IKKβ-dependent phosphorylation and ubiquitin-proteasome pathway (Karin and Ben-Neriah 2000). A recent study shows that NLRC5, a member of NLR family inhibits the NF-κB signalling pathway by interacting with IKKα and IKKβ and blocking their phosphorylation after LPS stimulation (Cui, Zhu et al. 2010). Accordingly, depletion of

NLRC5 enhanced the activation of NF-κB and its responsive genes, TNFα and IL-6. Similarly, depletion of CHC enhanced the activation of NF-κB and the secretion of IL-8 after *S.flexneri* infection or TNFα stimulation (data not shown). Although it is unclear whether CHC can also interact with and sequestrate the IKK complex in resting cells, depletion of IKKα drastically reduced CHC silencing-induced IκBα degradation and IL-8 secretion, suggesting CHC depletion-induced NF-κB activation is also IKKα dependent. Thus, it is plausible that CHC might inhibit basal IKK activation via the interaction with the IKK complex or an upstream NF-κB regulator in resting cells.

Inhibition of IKK activation by CHC-binding proteins

Protein interaction studies have revealed several proteins of endocytic pathway that play additional functions as inhibitors of NF-κB signalling pathways. Tom1 (target of Myb1) interacts with CHC and Tollip (Toll-interacting protein), forming a complex regulating endocytosis of IL-1 receptor (IL-1R) (Yamakami, Yoshimori et al. 2003; Brissoni, Agostini et al. 2006). In addition, Tom1 inhibits NF-κB activation in the IL-1R, TNFR, and TLR2/4 signalling pathways (Yamakami and Yokosawa 2004; Oglesby, Bray et al. 2010). Tollip forms a complex with IRAK (IL-1R-associated kinase) to block phosphorylation of IRAK, which prevents IKK and NF-κB activation upon stimulation of IL-1R (Burns, Clatworthy et al. 2000). However, functions of Tom1 and Tollip as inhibitors of NF-κB in resting cells were not clearly addressed in their studies. CHC interaction with and inhibition of IRAK in resting cells is unknown, but might be worth testing. A proteomics analysis designed to identify binding partners of CHC may help to understand the molecular mechanism by which CHC inhibits NF-κB activation in resting cells. In a proteomics analysis, IKAP (IκB kinase complex-associated protein) was identified as a component in the clathrin-containing vesicles although the physical

interaction between IKAP and clathrin was not tested (Borner, Harbour et al. 2006). An independent study showed that IKAP interacts with NIK and IKK complex and is a scaffold protein of the IKK complex (Cohen, Henzel et al. 1998). Whether IKAP and CHC interacts with each other and, if so, whether the interaction contributes to the regulation of NF-κB await further investigation.

Endocytosis-independent functions of clathrin heavy chain

Although NF-κB activation can be induced by many different mechanisms, little is known about the interconnection between endocytosis and regulation of NF-κB. Whether an inhibition of normal clathrin-mediated endocytosis (CME) can induce NF-κB activation was examined. Our data showed that depletion of μ2 subunit of AP2 complex (AP2M1) blocked transferrin-transferrin receptor internalization, a typical CME process, to a degree similar to CHC depletion, but failed to induce basal IκBα degradation and IL-8 secretion. This suggests that basal NF-κB activation by depletion of CHC was not merely attributed to the inhibition of endocytosis, and CHC might play an endocytosis-independent function as a negative regulator of NF-κB. However, we cannot rule out the possibility that the inhibition of CHC-dependent but AP2 complex-independent endocytosis might be implicated in NF-κB activation. Some endocytosis-independent functions were reported. A study showed that a small fraction of clathrin heavy chain (CHC) in the nucleus can contribute to the tumor suppressor p53-mediated gene expression by interacting with p53 and facilitating recruitment of transcriptional cofactors (Enari, Ohmori et al. 2006). Another study showed that CHC stabilizes spindle fibers to assist congression of chromosome during mitosis when endocytosis is in halt (Royle, Bright et al. 2005).

Although the exact action of CHC in the regulation of basal NF-κB activity remains to be further characterized, results presented in this dissertation suggest that CHC may play a new role as an inhibitor of NF-κB in resting cells, independently of endocytosis. Given the constitutive NF-κB activation and high level of IL-8 secretion by the depletion of CHC, CHC protein concentration might be an important factor in the regulation of basal IKK activity toward phosphorylation and degradation of IκB, further suggesting that a defect in CHC expression may be associated with chronic inflammatory disorders or cancers.

Collectively, results presented in this dissertation highlight the complexity of NF-κB regulation far from being elucidated. Nevertheless, the RNAi technique must be appreciated in helping us reveal previously unknown functions of the well-characterized proteins, IKKα and CHC. A large scale functional genomics-approach like a genome-wide RNAi screen will eventually help us better understand the complex NF-κB signalling network.

REFERENCES

Adli, M. and A. S. Baldwin (2006). "IKK-i/IKKepsilon controls constitutive, cancer cell-associated NF-kappaB activity via regulation of Ser-536 p65/RelA phosphorylation." J Biol Chem **281**(37): 26976-26984.

Adli, M., E. Merkhofer, et al. (2010). "IKKalpha and IKKbeta each function to regulate NF-kappaB activation in the TNF-induced/canonical pathway." PLoS One **5**(2): e9428.

Akira, S., S. Uematsu, et al. (2006). "Pathogen recognition and innate immunity." Cell **124**(4): 783-801.

Alkalay, I., A. Yaron, et al. (1995). "Stimulation-dependent I kappa B alpha phosphorylation marks the NF-kappa B inhibitor for degradation via the ubiquitin-proteasome pathway." Proc Natl Acad Sci U S A **92**(23): 10599-10603.

Amerik, A. Y. and M. Hochstrasser (2004). "Mechanism and function of deubiquitinating enzymes." Biochim Biophys Acta **1695**(1-3): 189-207.

Arbibe, L., D. W. Kim, et al. (2007). "An injected bacterial effector targets chromatin access for transcription factor NF-kappaB to alter transcription of host genes involved in immune responses." Nat Immunol **8**(1): 47-56.

Ashida, H., M. Kim, et al. (2010). "A bacterial E3 ubiquitin ligase IpaH9.8 targets NEMO/IKKgamma to dampen the host NF-kappaB-mediated inflammatory response." Nat Cell Biol **12**(1): 66-73; sup pp 61-69.

Beg, A. A., W. C. Sha, et al. (1995). "Constitutive NF-kappa B activation, enhanced granulopoiesis, and neonatal lethality in I kappa B alpha-deficient mice." Genes Dev **9**(22): 2736-2746.

Betts, J. C. and G. J. Nabel (1996). "Differential regulation of NF-kappaB2(p100) processing and control by amino-terminal sequences." Mol Cell Biol **16**(11): 6363-6371.

Bhavsar, A. P., J. A. Guttman, et al. (2007). "Manipulation of host-cell pathways by bacterial pathogens." Nature **449**(7164): 827-834.

Bird, T. A., K. Schooley, et al. (1997). "Activation of nuclear transcription factor NF-kappaB by interleukin-1 is accompanied by casein kinase II-mediated phosphorylation of the p65 subunit." J Biol Chem **272**(51): 32606-32612.

Bogoyevitch, M. A., K. R. Ngoei, et al. (2010). "c-Jun N-terminal kinase (JNK) signaling: recent advances and challenges." Biochim Biophys Acta **1804**(3): 463-475.

Boneca, I. G. (2005). "The role of peptidoglycan in pathogenesis." Curr Opin Microbiol **8**(1): 46-53.

Bonen, D. K., Y. Ogura, et al. (2003). "Crohn's disease-associated NOD2 variants share a signaling defect in response to lipopolysaccharide and peptidoglycan." Gastroenterology **124**(1): 140-146.

Bonizzi, G. and M. Karin (2004). "The two NF-kappaB activation pathways and their role in innate and adaptive immunity." Trends Immunol **25**(6): 280-288.

Boone, D. L., E. E. Turer, et al. (2004). "The ubiquitin-modifying enzyme A20 is required for termination of Toll-like receptor responses." Nat Immunol **5**(10): 1052-1060.

Borner, G. H., M. Harbour, et al. (2006). "Comparative proteomics of clathrin-coated vesicles." J Cell Biol **175**(4): 571-578.

Bourdet-Sicard, R., C. Egile, et al. (2000). "Diversion of cytoskeletal processes by Shigella during invasion of epithelial cells." Microbes Infect **2**(7): 813-819.

Bradley, J. R. and J. S. Pober (2001). "Tumor necrosis factor receptor-associated factors (TRAFs)." Oncogene **20**(44): 6482-6491.

Brissoni, B., L. Agostini, et al. (2006). "Intracellular trafficking of interleukin-1 receptor I requires Tollip." Curr Biol **16**(22): 2265-2270.

Brummelkamp, T. R., S. M. Nijman, et al. (2003). "Loss of the cylindromatosis tumour suppressor inhibits apoptosis by activating NF-kappaB." Nature **424**(6950): 797-801.

Burns, K., J. Clatworthy, et al. (2000). "Tollip, a new component of the IL-1RI pathway, links IRAK to the IL-1 receptor." Nat Cell Biol **2**(6): 346-351.

Buss, H., A. Dorrie, et al. (2004). "Constitutive and interleukin-1-inducible phosphorylation of p65 NF-{kappa}B at serine 536 is mediated by multiple protein kinases including I{kappa}B kinase (IKK)-{alpha}, IKK{beta}, IKK{epsilon}, TRAF family member-associated (TANK)-binding kinase 1 (TBK1), and an unknown kinase and couples p65 to TATA-binding protein-associated factor II31-mediated interleukin-8 transcription." J Biol Chem **279**(53): 55633-55643.

Chamaillard, M., M. Hashimoto, et al. (2003). "An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid." Nat Immunol **4**(7): 702-707.

Chen, G., M. H. Shaw, et al. (2009). "NOD-like receptors: role in innate immunity and inflammatory disease." Annu Rev Pathol **4**: 365-398.

Chen, Z., J. Hagler, et al. (1995). "Signal-induced site-specific phosphorylation targets I kappa B alpha to the ubiquitin-proteasome pathway." Genes Dev **9**(13): 1586-1597.

Chiu, Y. H., M. Zhao, et al. (2009). "Ubiquitin in NF-kappaB signaling." Chem Rev **109**(4): 1549-1560.

Chung, J. Y., Y. C. Park, et al. (2002). "All TRAFs are not created equal: common and distinct molecular mechanisms of TRAF-mediated signal transduction." J Cell Sci **115**(Pt 4): 679-688.

Claing, A., S. A. Laporte, et al. (2002). "Endocytosis of G protein-coupled receptors: roles of G protein-coupled receptor kinases and beta-arrestin proteins." Prog Neurobiol **66**(2): 61-79.

Cohen, L., W. J. Henzel, et al. (1998). "IKAP is a scaffold protein of the IkappaB kinase complex." Nature **395**(6699): 292-296.

Conner, S. D. and S. L. Schmid (2003). "Regulated portals of entry into the cell." Nature **422**(6927): 37-44.

Courtois, G. and T. D. Gilmore (2006). "Mutations in the NF-kappaB signaling pathway: implications for human disease." Oncogene **25**(51): 6831-6843.

Courtois, G., A. Smahi, et al. (2003). "A hypermorphic IkappaBalpha mutation is associated with autosomal dominant anhidrotic ectodermal dysplasia and T cell immunodeficiency." J Clin Invest **112**(7): 1108-1115.

Cui, J., L. Zhu, et al. (2010). "NLRC5 negatively regulates the NF-kappaB and type I interferon signaling pathways." Cell **141**(3): 483-496.

Dehio, C., M. C. Prevost, et al. (1995). "Invasion of epithelial cells by Shigella flexneri induces tyrosine phosphorylation of cortactin by a pp60c-src-mediated signalling pathway." EMBO J **14**(11): 2471-2482.

Dejardin, E. (2006). "The alternative NF-kappaB pathway from biochemistry to biology: pitfalls and promises for future drug development." Biochem Pharmacol **72**(9): 1161-1179.

Deng, L., C. Wang, et al. (2000). "Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain." Cell **103**(2): 351-361.

DiDonato, J., F. Mercurio, et al. (1996). "Mapping of the inducible IkappaB phosphorylation sites that signal its ubiquitination and degradation." Mol Cell Biol **16**(4): 1295-1304.

DiDonato, J. A., M. Hayakawa, et al. (1997). "A cytokine-responsive IkappaB kinase that activates the transcription factor NF-kappaB." Nature **388**(6642): 548-554.

Ding, S. Z., W. Fischer, et al. (2010). "Helicobacter pylori-induced histone modification, associated gene expression in gastric epithelial cells, and its implication in pathogenesis." PLoS One **5**(4): e9875.

Ducut Sigala, J. L., V. Bottero, et al. (2004). "Activation of transcription factor NF-kappaB requires ELKS, an IkappaB kinase regulatory subunit." Science **304**(5679): 1963-1967.

Enari, M., K. Ohmori, et al. (2006). "Requirement of clathrin heavy chain for p53-mediated transcription." Genes Dev **20**(9): 1087-1099.

Evans, P. C., H. Ovaa, et al. (2004). "Zinc-finger protein A20, a regulator of inflammation and cell survival, has de-ubiquitinating activity." Biochem J **378**(Pt 3): 727-734.

Fan, C. M. and T. Maniatis (1991). "Generation of p50 subunit of NF-kappa B by processing of p105 through an ATP-dependent pathway." Nature **354**(6352): 395-398.
Ferreiro, D. U. and E. A. Komives "Molecular mechanisms of system control of NF-kappaB signaling by IkappaBalpha." Biochemistry **49**(8): 1560-1567.
Gao, H., Y. Sun, et al. (2004). "Identification of beta-arrestin2 as a G protein-coupled receptor-stimulated regulator of NF-kappaB pathways." Mol Cell **14**(3): 303-317.
Geijtenbeek, T. B. and S. I. Gringhuis (2009). "Signalling through C-type lectin receptors: shaping immune responses." Nat Rev Immunol **9**(7): 465-479.
Gerondakis, S. and U. Siebenlist "Roles of the NF-kappaB pathway in lymphocyte development and function." Cold Spring Harb Perspect Biol **2**(5): a000182.
Ghosh, S. and M. S. Hayden (2008). "New regulators of NF-kappaB in inflammation." Nat Rev Immunol **8**(11): 837-848.
Gilmore, T. D. (2006). "Introduction to NF-kappaB: players, pathways, perspectives." Oncogene **25**(51): 6680-6684.
Girardin, S. E., I. G. Boneca, et al. (2003). "Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection." J Biol Chem **278**(11): 8869-8872.
Girardin, S. E., R. Tournebize, et al. (2001). "CARD4/Nod1 mediates NF-kappaB and JNK activation by invasive Shigella flexneri." EMBO Rep **2**(8): 736-742.
Goodridge, H. S., A. J. Wolf, et al. (2009). "Beta-glucan recognition by the innate immune system." Immunol Rev **230**(1): 38-50.
Hacker, H. and M. Karin (2006). "Regulation and function of IKK and IKK-related kinases." Sci STKE **2006**(357): re13.
Hamon, M. A., E. Batsche, et al. (2007). "Histone modifications induced by a family of bacterial toxins." Proc Natl Acad Sci U S A **104**(33): 13467-13472.
Harant, H., R. de Martin, et al. (1996). "Synergistic activation of interleukin-8 gene transcription by all-trans-retinoic acid and tumor necrosis factor-alpha involves the transcription factor NF-kappaB." J Biol Chem **271**(43): 26954-26961.
Hasegawa, M., Y. Fujimoto, et al. (2008). "A critical role of RICK/RIP2 polyubiquitination in Nod-induced NF-kappaB activation." Embo J **27**(2): 373-383.
Hayden, M. S. and S. Ghosh (2008). "Shared principles in NF-kappaB signaling." Cell **132**(3): 344-362.
Hess, J., P. Angel, et al. (2004). "AP-1 subunits: quarrel and harmony among siblings." J Cell Sci **117**(Pt 25): 5965-5973.
Heusch, M., L. Lin, et al. (1999). "The generation of nfkb2 p52: mechanism and efficiency." Oncogene **18**(46): 6201-6208.
Hideshima, T., D. Chauhan, et al. (2002). "NF-kappa B as a therapeutic target in multiple myeloma." J Biol Chem **277**(19): 16639-16647.
High, N., J. Mounier, et al. (1992). "IpaB of Shigella flexneri causes entry into epithelial cells and escape from the phagocytic vacuole." Embo J **11**(5): 1991-1999.
Hinz, M., M. Broemer, et al. (2007). "Signal responsiveness of IkappaB kinases is determined by Cdc37-assisted transient interaction with Hsp90." J Biol Chem **282**(44): 32311-32319.
Hoffmann, A., G. Natoli, et al. (2006). "Transcriptional regulation via the NF-kappaB signaling module." Oncogene **25**(51): 6706-6716.
Huang, B., X. D. Yang, et al. "Posttranslational modifications of NF-kappaB: another layer of regulation for NF-kappaB signaling pathway." Cell Signal **22**(9): 1282-1290.
Huang, W. C., J. J. Chen, et al. (2003). "c-Src-dependent tyrosine phosphorylation of IKKbeta is involved in tumor necrosis factor-alpha-induced intercellular adhesion molecule-1 expression." J Biol Chem **278**(11): 9944-9952.
Huang, W. C., J. J. Chen, et al. (2003). "Tyrosine phosphorylation of I-kappa B kinase alpha/beta by protein kinase C-dependent c-Src activation is involved in TNF-alpha-induced cyclooxygenase-2 expression." J Immunol **170**(9): 4767-4775.
Hugot, J. P., M. Chamaillard, et al. (2001). "Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease." Nature **411**(6837): 599-603.

Hysi, P., M. Kabesch, et al. (2005). "NOD1 variation, immunoglobulin E and asthma." Hum Mol Genet **14**(7): 935-941.

Inohara, Chamaillard, et al. (2005). "NOD-LRR proteins: role in host-microbial interactions and inflammatory disease." Annu Rev Biochem **74**: 355-383.

Inohara, N., T. Koseki, et al. (2000). "An induced proximity model for NF-kappa B activation in the Nod1/RICK and RIP signaling pathways." J Biol Chem **275**(36): 27823-27831.

Inohara, N. and G. Nunez (2003). "NODs: intracellular proteins involved in inflammation and apoptosis." Nat Rev Immunol **3**(5): 371-382.

Jacobs, M. D. and S. C. Harrison (1998). "Structure of an IkappaBalpha/NF-kappaB complex." Cell **95**(6): 749-758.

Janssen, R., A. van Wengen, et al. (2004). "The same IkappaBalpha mutation in two related individuals leads to completely different clinical syndromes." J Exp Med **200**(5): 559-568.

Jung, H. C., L. Eckmann, et al. (1995). "A distinct array of proinflammatory cytokines is expressed in human colon epithelial cells in response to bacterial invasion." J Clin Invest **95**(1): 55-65.

Kanayama, A., R. B. Seth, et al. (2004). "TAB2 and TAB3 activate the NF-kappaB pathway through binding to polyubiquitin chains." Mol Cell **15**(4): 535-548.

Karin, M. and Y. Ben-Neriah (2000). "Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity." Annu Rev Immunol **18**: 621-663.

Karin, M. and F. R. Greten (2005). "NF-kappaB: linking inflammation and immunity to cancer development and progression." Nat Rev Immunol **5**(10): 749-759.

Kato, H., O. Takeuchi, et al. (2008). "Length-dependent recognition of double-stranded ribonucleic acids by retinoic acid-inducible gene-I and melanoma differentiation-associated gene 5." J Exp Med **205**(7): 1601-1610.

Kawai, T. and S. Akira "The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors." Nat Immunol **11**(5): 373-384.

Kim, D. W., G. Lenzen, et al. (2005). "The Shigella flexneri effector OspG interferes with innate immune responses by targeting ubiquitin-conjugating enzymes." Proc Natl Acad Sci U S A **102**(39): 14046-14051.

Kirchhausen, T. (2000). "Clathrin." Annu Rev Biochem **69**: 699-727.

Kishore, N., C. Sommers, et al. (2003). "A selective IKK-2 inhibitor blocks NF-kappa B-dependent gene expression in interleukin-1 beta-stimulated synovial fibroblasts." J Biol Chem **278**(35): 32861-32871.

Kobayashi, Y. (2008). "The role of chemokines in neutrophil biology." Front Biosci **13**: 2400-2407.

Kopp, E. and S. Ghosh (1994). "Inhibition of NF-kappa B by sodium salicylate and aspirin." Science **265**(5174): 956-959.

Kovalenko, A., C. Chable-Bessia, et al. (2003). "The tumour suppressor CYLD negatively regulates NF-kappaB signalling by deubiquitination." Nature **424**(6950): 801-805.

Kray, A. E., R. S. Carter, et al. (2005). "Positive regulation of IkappaB kinase signaling by protein serine/threonine phosphatase 2A." J Biol Chem **280**(43): 35974-35982.

Krupnick, J. G., O. B. Goodman, Jr., et al. (1997). "Arrestin/clathrin interaction. Localization of the clathrin binding domain of nonvisual arrestins to the carboxy terminus." J Biol Chem **272**(23): 15011-15016.

Kuo, M. H. and C. D. Allis (1998). "Roles of histone acetyltransferases and deacetylases in gene regulation." Bioessays **20**(8): 615-626.

Kuper, H., H. O. Adami, et al. (2000). "Infections as a major preventable cause of human cancer." J Intern Med **248**(3): 171-183.

Lawrence, T. (2009). "The nuclear factor NF-kappaB pathway in inflammation." Cold Spring Harb Perspect Biol **1**(6): a001651.

Lawrence, T. and M. Bebien (2007). "IKKalpha in the regulation of inflammation and adaptive immunity." Biochem Soc Trans **35**(Pt 2): 270-272.

Lee, D. F. and M. C. Hung (2008). "Advances in targeting IKK and IKK-related kinases for cancer therapy." Clin Cancer Res **14**(18): 5656-5662.

Lefkowitz, R. J. and E. J. Whalen (2004). "beta-arrestins: traffic cops of cell signaling." Curr Opin Cell Biol **16**(2): 162-168.

Lewis, J., A. Devin, et al. (2000). "Disruption of hsp90 function results in degradation of the death domain kinase, receptor-interacting protein (RIP), and blockage of tumor necrosis factor-induced nuclear factor-kappaB activation." J Biol Chem **275**(14): 10519-10526.

Li, H., H. Xu, et al. (2007). "The phosphothreonine lyase activity of a bacterial type III effector family." Science **315**(5814): 1000-1003.

Lin, L. and S. Ghosh (1996). "A glycine-rich region in NF-kappaB p105 functions as a processing signal for the generation of the p50 subunit." Mol Cell Biol **16**(5): 2248-2254.

Lindner, R. and E. Ungewickell (1991). "Light-chain-independent binding of adaptors, AP180, and auxilin to clathrin." Biochemistry **30**(37): 9097-9101.

Liou, H. C. and D. Baltimore (1993). "Regulation of the NF-kappa B/rel transcription factor and I kappa B inhibitor system." Curr Opin Cell Biol **5**(3): 477-487.

Loo, Y. M., J. Fornek, et al. (2008). "Distinct RIG-I and MDA5 signaling by RNA viruses in innate immunity." J Virol **82**(1): 335-345.

Luan, B., Z. Zhang, et al. (2005). "Beta-arrestin2 functions as a phosphorylation-regulated suppressor of UV-induced NF-kappaB activation." EMBO J **24**(24): 4237-4246.

Martinon, F. and J. Tschopp (2005). "NLRs join TLRs as innate sensors of pathogens." Trends Immunol **26**(8): 447-454.

May, M. J., F. D'Acquisto, et al. (2000). "Selective inhibition of NF-kappaB activation by a peptide that blocks the interaction of NEMO with the IkappaB kinase complex." Science **289**(5484): 1550-1554.

Medzhitov, R. (2007). "Recognition of microorganisms and activation of the immune response." Nature **449**(7164): 819-826.

Menard, R., M. C. Prevost, et al. (1996). "The secreted Ipa complex of Shigella flexneri promotes entry into mammalian cells." Proc Natl Acad Sci U S A **93**(3): 1254-1258.

Mercurio, F., H. Zhu, et al. (1997). "IKK-1 and IKK-2: cytokine-activated IkappaB kinases essential for NF-kappaB activation." Science **278**(5339): 860-866.

Mounier, J., V. Laurent, et al. (1999). "Rho family GTPases control entry of Shigella flexneri into epithelial cells but not intracellular motility." J Cell Sci **112 (Pt 13)**: 2069-2080.

Mousavi, S. A., L. Malerod, et al. (2004). "Clathrin-dependent endocytosis." Biochem J **377**(Pt 1): 1-16.

Muegge, K. (2002). "Preparing the target for the bullet." Nat Immunol **3**(1): 16-17.

Mukaida, N., Y. Mahe, et al. (1990). "Cooperative interaction of nuclear factor-kappa B- and cis-regulatory enhancer binding protein-like factor binding elements in activating the interleukin-8 gene by pro-inflammatory cytokines." J Biol Chem **265**(34): 21128-21133.

Munshi, A., G. Shafi, et al. (2009). "Histone modifications dictate specific biological readouts." J Genet Genomics **36**(2): 75-88.

Oglesby, I. K., I. M. Bray, et al. (2010). "miR-126 is downregulated in cystic fibrosis airway epithelial cells and regulates TOM1 expression." J Immunol **184**(4): 1702-1709.

Okamoto, T. (2006). "NF-kappaB and rheumatic diseases." Endocr Metab Immune Disord Drug Targets **6**(4): 359-372.

Owen, D. J., B. M. Collins, et al. (2004). "Adaptors for clathrin coats: structure and function." Annu Rev Cell Dev Biol **20**: 153-191.

Pahl, H. L. (1999). "Activators and target genes of Rel/NF-kappaB transcription factors." Oncogene **18**(49): 6853-6866.

Palanki, M. S. (2002). "Inhibitors of AP-1 and NF-kappa B mediated transcriptional activation: therapeutic potential in autoimmune diseases and structural diversity." Curr Med Chem **9**(2): 219-227.

Palkowitsch, L., J. Leidner, et al. (2008). "Phosphorylation of serine 68 in the IkappaB kinase (IKK)-binding domain of NEMO interferes with the structure of the IKK complex and tumor necrosis factor-alpha-induced NF-kappaB activity." J Biol Chem **283**(1): 76-86.

Park, J. H., Y. G. Kim, et al. (2007). "RICK/RIP2 mediates innate immune responses induced through Nod1 and Nod2 but not TLRs." J Immunol **178**(4): 2380-2386.

Perdomo, O. J., J. M. Cavaillon, et al. (1994). "Acute inflammation causes epithelial invasion and mucosal destruction in experimental shigellosis." J Exp Med **180**(4): 1307-1319.

Perkins, N. D. (2006). "Post-translational modifications regulating the activity and function of the nuclear factor kappa B pathway." Oncogene **25**(51): 6717-6730.

Philpott, D. J., S. Yamaoka, et al. (2000). "Invasive Shigella flexneri activates NF-kappa B through a lipopolysaccharide-dependent innate intracellular response and leads to IL-8 expression in epithelial cells." J Immunol **165**(2): 903-914.

Pickart, C. M. and M. J. Eddins (2004). "Ubiquitin: structures, functions, mechanisms." Biochim Biophys Acta **1695**(1-3): 55-72.

Poyet, J. L., S. M. Srinivasula, et al. (2000). "Activation of the Ikappa B kinases by RIP via IKKgamma /NEMO-mediated oligomerization." J Biol Chem **275**(48): 37966-37977.

Proell, M., S. J. Riedl, et al. (2008). "The Nod-like receptor (NLR) family: a tale of similarities and differences." PLoS One **3**(4): e2119.

Reiter, E. and R. J. Lefkowitz (2006). "GRKs and beta-arrestins: roles in receptor silencing, trafficking and signaling." Trends Endocrinol Metab **17**(4): 159-165.

Robinson, M. J., F. Osorio, et al. (2009). "Dectin-2 is a Syk-coupled pattern recognition receptor crucial for Th17 responses to fungal infection." J Exp Med **206**(9): 2037-2051.

Rohde, J. R., A. Breitkreutz, et al. (2007). "Type III secretion effectors of the IpaH family are E3 ubiquitin ligases." Cell Host Microbe **1**(1): 77-83.

Royle, S. J., N. A. Bright, et al. (2005). "Clathrin is required for the function of the mitotic spindle." Nature **434**(7037): 1152-1157.

Sakurai, H., H. Chiba, et al. (1999). "IkappaB kinases phosphorylate NF-kappaB p65 subunit on serine 536 in the transactivation domain." J Biol Chem **274**(43): 30353-30356.

Sansonetti, P. J. (1998). "Pathogenesis of shigellosis: from molecular and cellular biology of epithelial cell invasion to tissue inflammation and vaccine development." Jpn J Med Sci Biol **51 Suppl**: S69-80.

Sansonetti, P. J., G. Tran Van Nhieu, et al. (1999). "Rupture of the intestinal epithelial barrier and mucosal invasion by Shigella flexneri." Clin Infect Dis **28**(3): 466-475.

Schroeder, G. N. and H. Hilbi (2008). "Molecular pathogenesis of Shigella spp.: controlling host cell signaling, invasion, and death by type III secretion." Clin Microbiol Rev **21**(1): 134-156.

Sen, R. and D. Baltimore (1986). "Inducibility of kappa immunoglobulin enhancer-binding protein Nf-kappa B by a posttranslational mechanism." Cell **47**(6): 921-928.

Shenoy, S. K., M. T. Drake, et al. (2006). "beta-arrestin-dependent, G protein-independent ERK1/2 activation by the beta2 adrenergic receptor." J Biol Chem **281**(2): 1261-1273.

Sizemore, N., S. Leung, et al. (1999). "Activation of phosphatidylinositol 3-kinase in response to interleukin-1 leads to phosphorylation and activation of the NF-kappaB p65/RelA subunit." Mol Cell Biol **19**(7): 4798-4805.

Skaug, B., X. Jiang, et al. (2009). "The role of ubiquitin in NF-kappaB regulatory pathways." Annu Rev Biochem **78**: 769-796.

Solt, L. A. and M. J. May (2008). "The IkappaB kinase complex: master regulator of NF-kappaB signaling." Immunol Res **42**(1-3): 3-18.

Spencer, E., J. Jiang, et al. (1999). "Signal-induced ubiquitination of IkappaBalpha by the F-box protein Slimb/beta-TrCP." Genes Dev **13**(3): 284-294.

Tada, K., T. Okazaki, et al. (2001). "Critical roles of TRAF2 and TRAF5 in tumor necrosis factor-induced NF-kappa B activation and protection from cell death." J Biol Chem **276**(39): 36530-36534.

Takeuchi, O. and S. Akira "Pattern recognition receptors and inflammation." Cell **140**(6): 805-820.

Takeuchi, O. and S. Akira (2009). "Innate immunity to virus infection." Immunol Rev **227**(1): 75-86.

Thomson, S., A. L. Clayton, et al. (1999). "The nucleosomal response associated with immediate-early gene induction is mediated via alternative MAP kinase cascades: MSK1 as a potential histone H3/HMG-14 kinase." EMBO J **18**(17): 4779-4793.

Tran Van Nhieu, G., A. Ben-Ze'ev, et al. (1997). "Modulation of bacterial entry into epithelial cells by association between vinculin and the Shigella IpaA invasin." Embo J **16**(10): 2717-2729.

Tran Van Nhieu, G., R. Bourdet-Sicard, et al. (2000). "Bacterial signals and cell responses during Shigella entry into epithelial cells." Cell Microbiol **2**(3): 187-193.

Tran Van Nhieu, G., E. Caron, et al. (1999). "IpaC induces actin polymerization and filopodia formation during Shigella entry into epithelial cells." Embo J **18**(12): 3249-3262.

Trompouki, E., E. Hatzivassiliou, et al. (2003). "CYLD is a deubiquitinating enzyme that negatively regulates NF-kappaB activation by TNFR family members." Nature **424**(6950): 793-796.

Wang, C., L. Deng, et al. (2001). "TAK1 is a ubiquitin-dependent kinase of MKK and IKK." Nature **412**(6844): 346-351.

Wang, Y., Y. Tang, et al. (2006). "Association of beta-arrestin and TRAF6 negatively regulates Toll-like receptor-interleukin 1 receptor signaling." Nat Immunol **7**(2): 139-147.

Wen, D., Y. Nong, et al. (2006). "A selective small molecule IkappaB Kinase beta inhibitor blocks nuclear factor kappaB-mediated inflammatory responses in human fibroblast-like synoviocytes, chondrocytes, and mast cells." J Pharmacol Exp Ther **317**(3): 989-1001.

Wen, X. and G. D. Wu (2001). "Evidence for epigenetic mechanisms that silence both basal and immune-stimulated transcription of the IL-8 gene." J Immunol **166**(12): 7290-7299.

Wertz, I. E., K. M. O'Rourke, et al. (2004). "De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-kappaB signalling." Nature **430**(7000): 694-699.

Wilson, A. G. (2008). "Epigenetic regulation of gene expression in the inflammatory response and relevance to common diseases." J Periodontol **79**(8 Suppl): 1514-1519.

Winston, J. T., P. Strack, et al. (1999). "The SCFbeta-TRCP-ubiquitin ligase complex associates specifically with phosphorylated destruction motifs in IkappaBalpha and beta-catenin and stimulates IkappaBalpha ubiquitination in vitro." Genes Dev **13**(3): 270-283.

Witherow, D. S., T. R. Garrison, et al. (2004). "beta-Arrestin inhibits NF-kappaB activity by means of its interaction with the NF-kappaB inhibitor IkappaBalpha." Proc Natl Acad Sci U S A **101**(23): 8603-8607.

Woronicz, J. D., X. Gao, et al. (1997). "IkappaB kinase-beta: NF-kappaB activation and complex formation with IkappaB kinase-alpha and NIK." Science **278**(5339): 866-869.

Wu, C. J., D. B. Conze, et al. (2006). "Sensing of Lys 63-linked polyubiquitination by NEMO is a key event in NF-kappaB activation [corrected]." Nat Cell Biol **8**(4): 398-406.

Yamakami, M. and H. Yokosawa (2004). "Tom1 (target of Myb 1) is a novel negative regulator of interleukin-1- and tumor necrosis factor-induced signaling pathways." Biol Pharm Bull **27**(4): 564-566.

Yamakami, M., T. Yoshimori, et al. (2003). "Tom1, a VHS domain-containing protein, interacts with tollip, ubiquitin, and clathrin." J Biol Chem **278**(52): 52865-52872.

Yamamoto, Y., U. N. Verma, et al. (2003). "Histone H3 phosphorylation by IKK-alpha is critical for cytokine-induced gene expression." Nature **423**(6940): 655-659.

Yamasaki, S., E. Ishikawa, et al. (2008). "Mincle is an ITAM-coupled activating receptor that senses damaged cells." Nat Immunol **9**(10): 1179-1188.

Ybe, J. A., B. Greene, et al. (1998). "Clathrin self-assembly is regulated by three light-chain residues controlling the formation of critical salt bridges." EMBO J **17**(5): 1297-1303.

Zandi, E., D. M. Rothwarf, et al. (1997). "The IkappaB kinase complex (IKK) contains two kinase subunits, IKKalpha and IKKbeta, necessary for IkappaB phosphorylation and NF-kappaB activation." Cell **91**(2): 243-252.

Zhong, H., R. E. Voll, et al. (1998). "Phosphorylation of NF-kappa B p65 by PKA stimulates transcriptional activity by promoting a novel bivalent interaction with the coactivator CBP/p300." Mol Cell **1**(5): 661-671.

Zurawski, D. V., K. L. Mumy, et al. (2009). "Shigella flexneri type III secretion system effectors OspB and OspF target the nucleus to downregulate the host inflammatory response via interactions with retinoblastoma protein." Mol Microbiol **71**(2): 350-368.

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PLoS one

IKKα Contributes to Canonical NF-κB Activation Downstream of Nod1-Mediated Peptidoglycan Recognition

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Abstract

Background: During pathogen infection, innate immunity is initiated via the recognition of microbial products by pattern recognition receptors and the subsequent activation of transcription factors that upregulate proinflammatory genes. By controlling the expression of cytokines, chemokines, anti-bacterial peptides and adhesion molecules, the transcription factor nuclear factor-kappa B (NF-κB) has a central function in this process. In a typical model of NF-κB activation, the recognition of pathogen associated molecules triggers the canonical NF-κB pathway that depends on the phosphorylation of Inhibitor of NF-κB (IκB) by the catalytic subunit IκB kinase β (IKKβ), its degradation and the nuclear translocation of NF-κB dimers.

Methodology: Here, we performed an RNA interference (RNAi) screen on *Shigella flexneri*-induced NF-κB activation to identify new factors involved in the regulation of NF-κB following infection of epithelial cells by invasive bacteria. By targeting a subset of the human signaling proteome, we found that the catalytic subunit IKKα is also required for complete NF-κB activation during infection. Depletion of IKKα by RNAi strongly reduces the nuclear translocation of NF-κB p65 during *S. flexneri* infection as well as the expression of the proinflammatory chemokine interleukin-8. Similar to IKKβ, IKKα contributes to the phosphorylation of IκBα on serines 32 and 36, and to its degradation. Experiments performed with the synthetic Nod1 ligand L-Ala-D-γ-Glu-meso-diaminopimelic acid confirmed that IKKα is involved in NF-κB activation triggered downstream of Nod1-mediated peptidoglycan recognition.

Conclusions: Taken together, these results demonstrate the unexpected role of IKKα in the canonical NF-κB pathway triggered by peptidoglycan recognition during bacterial infection. In addition, they suggest that IKKα may be an important drug target for the development of treatments that aim at limiting inflammation in bacterial infection.

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Introduction

During pathogen infection, structurally conserved microbial molecules are recognized by germline-encoded pathogen recognition receptors (PRRs) that function as sensors for non-self detection and initiate innate immunity [1,2]. PRRs include transmembrane proteins such as Toll-like receptors and C-type lectin receptors, as well as cytoplasmic proteins such as retinoic acid-inducible gene (RIG)-I-like receptors and NOD-like receptors [3,4,5]. They are expressed in macrophages and dendritic cells but also in various non-professional immune cells including epithelial and endothelial cells. PRRs recognize a large variety of pathogen associated molecular patterns (PAMPs) from both extracellular and intracellular pathogens including lipopolysaccharide, peptidoglycan, lipoproteins, dsRNA, ssRNA, CpG-DNA and flagellin [6]. Signaling pathways of PAMP recognition converge into the activation of the pleiotropic transcription factor nuclear factor-kappa B (NF-κB) that, in the context of innate immunity, regulates the expression of proinflammatory genes encoding cytokines, chemokines, anti-bacterial peptides and adhesion molecules [7]. The mammalian NF-κB family consists of the members RelA/p65, RelB, c-Rel, p50 (NF-κB1) and p52 (NF-κB2) [8]. All five proteins share a Rel homology domain and form homo- and heterodimers that regulate transcription by binding to κB sites in promoters or enhancers of target genes. In unstimulated cells, most of the NF-κB dimers are sequestrated in the cytoplasm by the proteins of the Inhibitor of NF-κB (IκB) family whose prototype is IκBα. In the canonical pathway of NF-κB activation triggered by most stimuli including bacterial and viral infection, cytokines and stress-induced responses, phosphorylation of IκBα on Serine 32 and Serine 36 residues by the IκB kinase (IKK) complex is a decisive regulatory step [9]. The IKK complex is comprised of three subunits: two catalytic subunits, IKKα and IKKβ, and the regulatory scaffold component NF-κB essential modulator (NEMO). The respective contribution of IKKα and IKKβ in the phosphorylation of IκBα is unclear. Although it is generally accepted that IKKβ is critical for IκBα phosphorylation through the canonical pathway, two recent reports demonstrate the

equal importance of IKKα for the activation of NF-κB by the inflammatory cytokines interleukin-1 (IL-1) in mouse embryonic fibroblasts and tumor necrosis factor alpha (TNFα) in HeLa cells [10,11]. The phosphorylation of IκBα is followed by its rapid polyubiquitination and subsequent degradation by the 26S proteasome complex [12]. The release of NF-κB with unmasked nuclear localization sequence leads then to the translocation of the transcription factor to the nucleus where it regulates gene expression [13].

Although the role of NF-κB is central to many pathways triggered by pathogen recognition, the molecular processes that govern its activation are only partially elucidated. In particular, the mechanisms triggered by the detection of invasive bacteria such as the pathogen *Shigella flexneri* remain largely uncharacterized. *S. flexneri* makes use of a type III secretion (T3S) apparatus to locally rearrange the host actin cytoskeleton and penetrate into intestinal epithelial cells [14]. Once internalized, bacteria multiply in the host cytoplasm and use actin-based motility to spread to adjacent epithelial cells. During infection, massive inflammation is observed in colonic mucosal tissues [15]. In infected epithelial cells, intracellular bacteria release peptidoglycan-derived peptides that are specifically recognized by Nod1 [16]. Upon ligand binding, Nod1 homo-dimerizes and recruits the downstream kinase RICK/RIPK2 through heterologous caspase-recruitment domain interactions [17]. This converges to the sequential recruitment and activation of the TAK1/TAB1/TAB2 and IKKα/IKKβ/IKKγ complexes, the nuclear translocation of NF-κB and the upregulation of proinflammatory genes encoding for cytokines and chemokines, including interleukin-8 (IL-8) and TNFα [18]. The chemokine IL-8 recruits polymorphonuclear cells to the site of infection and therefore contributes to contain the dissemination of bacteria within the intestinal tissue. Interestingly, *S. flexneri* uses the T3S apparatus to secrete several effectors that alter multiple signaling pathways in infected cells and reduce the expression of proinflammatory genes [19]. Among others, the effector OspF suppresses the expression of IL-8 by dephosphorylating the MAP kinases p38 and ERK in the nucleus of infected cells [20,21], thereby impairing the phosphorylation of Histone H3, a process that regulates the access of chromatin to transcription factors.

Here, we performed an RNA interference (RNAi) screen on *S. flexneri*-induced NF-κB activation to identify new factors involved in the regulation of NF-κB following infection of epithelial cells by invasive bacteria. By targeting a subset of the human signaling proteome, we identified IKKα as a protein required for *S. flexneri*-induced NF-κB nuclear translocation and IL-8 secretion in HeLa cells. This result was unexpected because, except for IL-1 and TNFα [10,11], it is generally accepted that IKKβ is the component of the IKK complex involved in the canonical pathway of NF-κB activation. Depletion of IKKα or IKKβ indicated that *S. flexneri*-induced NF-κB activation in HeLa cells requires indeed both catalytic subunits. We further characterized the role of IKKα and found that, during *S. flexneri* infection, IKKα was required for the phosphorylation of IκBα on serines 32 and 36, and for its degradation. Experiments performed with the synthetic Nod1 ligand L-Ala-D-γ-Glu-meso-diaminopimelic acid (Tri-DAP) indicated that IKKα was involved in Nod1-mediated signaling pathway of NF-κB activation. Taken together, these results show that, although Nod1 signaling triggers the canonical pathway of NF-κB activation, both IKKα and IKKβ are required for full NF-κB activation.

Materials and Methods

Antibodies and reagents

Antibodies against NF-κB p65, IκBα and IKKα were obtained from Santa Cruz Biotechnology (Santa Cruz, USA) whereas the anti-actin was from Chemicon (Billerica, USA) and the anti-phospho-IκBα was from Cell signaling technology (Beverly, USA). The anti-mouse IgG-Cy5 was obtained from Zymed (San Francisco, USA) and the anti-rabbit IgG-HRP and anti-mouse IgG-HRP from GE Healthcare (Pittsburgh, USA). Hoechst and FITC-phalloidin were from Invitrogen (Carlsbad, USA), TNFα from R & D systems (Minneapolis, USA).

Cell culture and transfection

HeLa cells were maintained in Dulbecco's modified Eagle's medium (high glucose) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in 10% CO_2. HeLa cells were transfected with siRNAs and DNA plasmids using Lipofectamine 2000 (Invitrogen, Carlsbad, USA) and jetPEI (Poly plus transfection, Illkirch, France), respectively. siRNAs ON-TARGETplus SMARTpool targeting IKKα (#L-003473-00-005) and ON-TARGETplus siCONTROL (Dharmacon, Dallas, USA) were used in all our study except for the experiments where IKKα, IKKβ and NEMO were silenced in parallel. In this case, all siRNAs were from Qiagen (Valencia, CA, USA).

In vitro diced siRNA library

An *in vitro* diced siRNA library targeting 132 genes coding for a subset of the signaling proteome was generated as previously described [22,23,24]. Briefly, for each gene, a 600 base pair cDNA was generated by PCR from a total cDNA library. An additional set of nested primers was used to add T7 promoters at both ends of the final cDNA fragment. Nested PCR products were subject to *in vitro* transcription, dicing, and purification to produce gene specific siRNA pools. Dicing was performed with the turbo dicer siRNA generation kit from Genlantis (San Diego, USA). The concentration of all siRNA pools was normalized.

Bacterial strains

The *S. flexneri* strains M90T wild-type and the *icsA* (*virG*) deletion mutant (Δ*virG*) were generously provided by Dr. P. Sansonetti (Institut Pasteur, Paris, France). All strains were transformed with the pMW211 plasmid to express the DsRed protein under control of a constitutive promoter. The pMW211 plasmid was a generous gift from Dr. D. Bumann (Biozentrum, University of Basel, Switzerland). The Δ*ospF* deletion mutant used in IL-8 expression experiments, was generated from the Δ*virG* mutant by allelic exchange using a modification of the lambda red-mediated gene deletion [25]. Briefly, the genes for lambda red recombination were expressed from the pKM208 plasmid [26]. The chloramphenicol resistance cassette (cat) of the pKD3 plasmid was amplified using the primers listed in Table 1. After DpnI digestion, the PCR product was electroporated into the Δ*virG* mutant. Recombinants were selected on TSB plates containing 5 or 10 μg ml^{-1} chloramphenicol. The cat cassette was removed by transformation of pCP20 and incubation at 30°C on TSB plates containing 100 μg ml^{-1} ampicillin [25]. Single colonies were screened by PCR.

Infection assay

Bacteria were routinely grown in tryptic soy broth (TSB) medium, used in exponential growth phase, and treated with poly-L-lysine prior infection. HeLa cells, seeded in 96-well plates, were serum starved for 30 min and infected with *S. flexneri* at a multiplicity of infection (MOI) of 10. Immediately after adding bacteria, the plates were centrifuged for 5 min at 2000 rpm and placed at 37°C for 30 min. Extracellular bacteria were killed by addition of gentamycin (50 μg/ml).

Table 1. Oligonucleotide primers used to generate the Δ*ospF* mutant.

Mutant	Forward	Reverse
Δ*ospF*	ATTCTATTATATAGATAAAATATCTCCTGCAAAAGATACGGGTAT TTTTGTGTAGGCTGGAGCTGCTTCG	TCAAAAGTTCGATGTTCCACCACATCGACCGTAGAAGAGATGAGATA GTACATATGAATATCCTCCTTAG

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Immunofluorescence

Cells were fixed with 4% PFA for 6 min and permeabilized in 0.5% Triton X-100 for 10 min. They were, then, incubated with a mouse monoclonal anti-p65 antibody (1 μg/ml) overnight at 4°C and stained with a Cy5-conjugated secondary antibody and Hoechst (10 μg/ml) for 40 min at room temperature.

siRNA screen of *S. flexneri*-induced p65 nuclear translocation

The *in vitro* diced siRNA library was screened on *S. flexneri*-induced p65 translocation assay in a 96-well format. The firefly *luciferase* (GL3) siRNA was used as a non-silencing negative control as described previously [22,23,24]. siRNA pools against Nod1, RIPK2 and Src were used as positive controls. The screen was performed three times in duplicate as follows. Three thousand HeLa cells per well were transfected by reverse transfection with the individual 132 siRNA pools in 96-well plates. After 48 hours, cells were infected with DsRed *S. flexneri* at MOI of 10 for 90 min and then fixed, permeabilized, and stained for p65, F-actin, and DNA. Images were acquired at 12 random sites of each well using the automated ImageXpress microscope (Molecular devices, Sunnyvale, USA). At each site, images at 360 nm, 480 nm, 594 nm, 640 nm were acquired to visualize Hoechst, Phalloidin, DsRed *S. flexneri* and p65, respectively. The nuclear localization of p65 was automatically quantified by using the Enhanced-Translocation module of MetaXpress (Molecular devices, Sunnyvale, USA). Briefly, the Hoechst staining was used as a mask to automatically identify nuclei in the p65 staining image. The cytoplasmic area was defined by a ring around each nucleus. For each cell, the ratio of p65 intensity in the nucleus and in the cytoplasmic ring defined as the Nuc/Cyt p65 ratio was calculated and averaged over several thousands of cells per well. The results of the screen were expressed as individual scores. The score of a particular gene represents the fold standard deviation from the mean of the GL3 control wells. A negative or a positive sign was assigned to the score when the Nuc/Cyt p65 ratio was lower or higher than the GL3 control ratio, respectively.

Enzyme- linked Immunosorbent Assay (ELISA)

IL-8 secretion was measured by ELISA in the supernatant of HeLa cells 6 hours post infection. Cell-free supernatants from triplicate wells were analyzed for their IL-8 content using a commercial ELISA kit (BD Pharmingen, San Jose, USA).

Western blot analysis

HeLa cells were transfected with siRNAs in a 6-well plate. 72 hours post transfection, cells were lysed in Phosphosafe Extraction Buffer (Novagen, Darmstadt, Germany) supplemented with 1x protease inhibitor cocktail (Calbiochem, Darmstadt, Germany). Protein concentration was measured using the bicinchonic acid (BCA) kit (Pierce, Rockford, USA). Equal amounts of proteins were resolved by SDS-PAGE and transferred to Hybond C-Extra membrane (Amersham Bioscience, Pittsburgh, USA) for immunoblotting with individual antibodies. Primary antibodies were detected using horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibodies, and visualized with the ECL system (Pierce). Quantification of the blots was performed using the densitometry feature of Photoshop.

Tri-DAP treatment

Cells were serum starved 30 min before treatment with L-Ala-γ-D-Glu-mesoDAP (Tri-DAP). Tri-DAP treatment was performed by calcium phosphate transfection using 0.4 μg/ml Tri-DAP final concentration for 90 min. Then cells were fixed with 4% PFA and analyzed by immunofluorescence as described above.

Statistical analysis

Data are expressed as mean ± standard deviation calculated from the number of replicates specified in figure legends. p values were calculated with a two-tailed two-sample equal variance t-test.

Results

RNAi screen identifies the role of IKKα in the nuclear translocation of NF-κB p65 during infection of epithelial cells by *S. flexneri*

Over the past 10 years, RNAi has been an essential tool to study gene function in mammalian cells [24,27,28]. Using *S. flexneri*-induced NF-κB activation in HeLa cells as a model system, we performed an image-based RNAi screen to identify new proteins involved in the molecular mechanisms that control NF-κB activation following pathogen recognition. The nuclear localization of NF-κB p65 visualized by anti-p65 immunofluorescence microscopy was used as readout for NF-κB activation. In uninfected cells, p65 was mainly localized in the cytoplasm (Figure 1A, left panel). In contrast, following infection with dsRed-expressing *S. flexneri*, a strong nuclear translocation of p65 was observed (Figure 1A, right panel). To validate the RNAi approach, silencing of the intracellular pattern recognition receptor Nod1 involved in *S. flexneri* recognition was tested. HeLa cells were transfected with pools of *in vitro* diced small interference RNA (siRNA) targeting Nod1 or the firefly *Luciférase* used as control (GL3). After 48 hours, cells were infected with *S. flexneri*, stained for p65 and DNA with an anti-p65 antibody and Hoechst, respectively. Visual inspection of images showed that *S. flexneri*-induced p65 nuclear translocation was suppressed in Nod1-depleted cells (Figure 1B). This observation was quantified by measuring for each cell the ratio of p65 intensity in the nucleus and in the cytoplasm by automated image processing (Figure 1C). The Hoechst staining was used to automatically identify nuclei whereas the cytoplasmic area was defined by a ring around each nucleus. Quantification of the Nucleus/Cytoplasm p65 intensity ratio (Nuc/Cyt p65 ratio) confirmed that the depletion of Nod1 inhibited the nuclear translocation of p65 induced during infection (Figure 1D), and therefore, that the RNAi approach was suitable to identify new proteins involved in the activation of NF-κB during *S. flexneri* infection of epithelial cells.

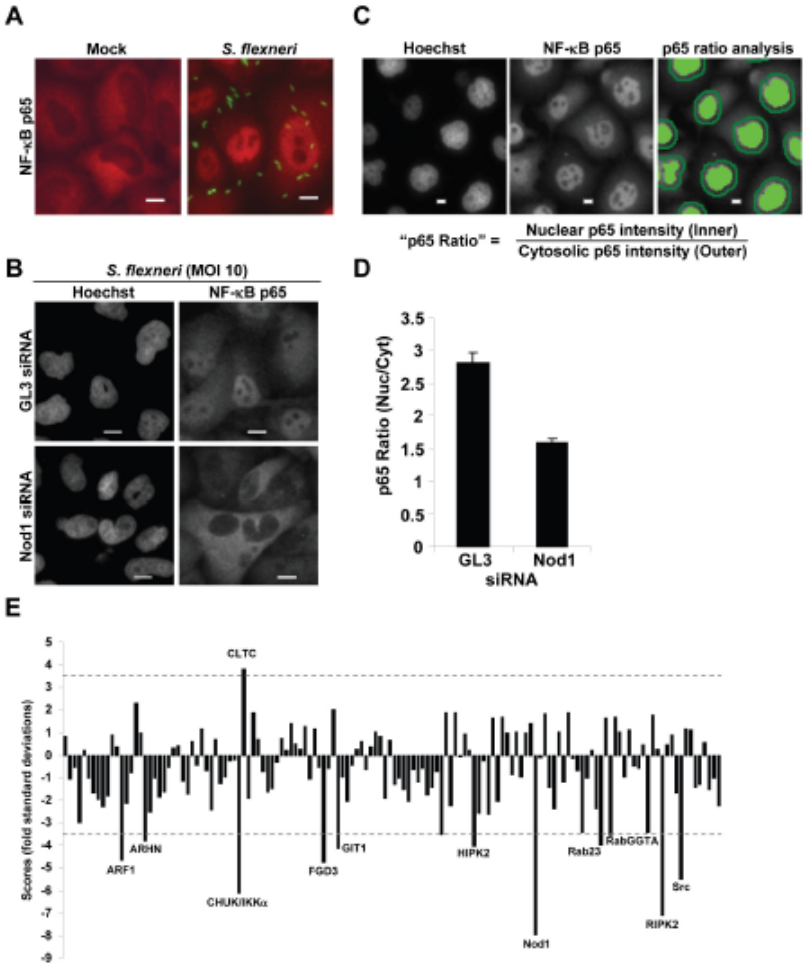

Figure 1. RNAi screen identifies the role of IKKα in p65 nuclear translocation during *S. flexneri* infection. (A) NF-κB p65 translocates to the nucleus in response to *S. flexneri* infection. HeLa cells were left untreated (Mock) or infected with dsRed-expressing *S. flexneri* for 60 minutes at MOI = 10. NF-κB p65 localization was visualized by immunofluorescence microscopy. An overlay image is shown for *S. flexneri* (green) and p65 (red). (B) Depletion of Nod1 by RNAi inhibits the nuclear translocation of NF-κB p65 in response to *S. flexneri* infection. HeLa cells were transfected with GL3 or Nod1 siRNA and infected with *S. flexneri* for 60 minutes at MOI = 10. p65 and DNA were visualized by anti-p65 and Hoechst staining, respectively. (C) Images illustrating the quantification of the p65 ratio. The Hoechst staining was used as a mask to automatically identify the nuclei in the p65 staining image. The cytoplasmic area was defined by a ring surrounding each nucleus. Scale bars, 10 μm (D) Quantification of the p65 ratio in control and Nod1-depleted cells following infection by *S. flexneri*. Results represent the mean ± SD of 12 images; graph representative of 3 independent experiments. (E) RNAi screen of *S. flexneri*-induced p65 nuclear translocation in HeLa cells. Scores are fold standard deviations from the mean of GL3 control wells. Dashed lines represent scores of ±3.5 (See Materials and Methods for details).
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An *in vitro* diced siRNA library targeting 132 genes from the human signaling proteome was screened on *S. flexneri*-induced p65 nuclear translocation as described in Materials and Methods. For each gene, a score representing the fold standard deviation from the mean of GL3 control wells was calculated (Figures 1E and Table S1). A negative or a positive sign was attributed to the score when the Nuc/Cyt p65 ratio was lower or higher than the GL3 ratio, respectively. As expected, Nod1 and RIPK2, two key

proteins involved in *S. flexneri*-induced NF-κB activation [16,29], as well as Src, a tyrosine kinase required for bacterial entry into cells [30], obtained strong negative scores (Figures 1E and Table S1). Using an arbitrarily determined cut off score value of +/− 3.5, the proteins CHUK/IKKα, FGD3, Arf1, GIT1, Rab23, ARHN, RabGGTA, HIPK2 and CLTC were classified as hits (Figures 1E and Table S1). The identification of IKKα, also known as CHUK, was unexpected as peptidoglycan recognition via Nod1 triggers the canonical NF-κB pathway, and was, therefore, thought to be exclusively dependent on IKKβ and NEMO. The contribution of IKKα in the mechanisms that control NF-κB activation following *S. flexneri* infection was then further explored.

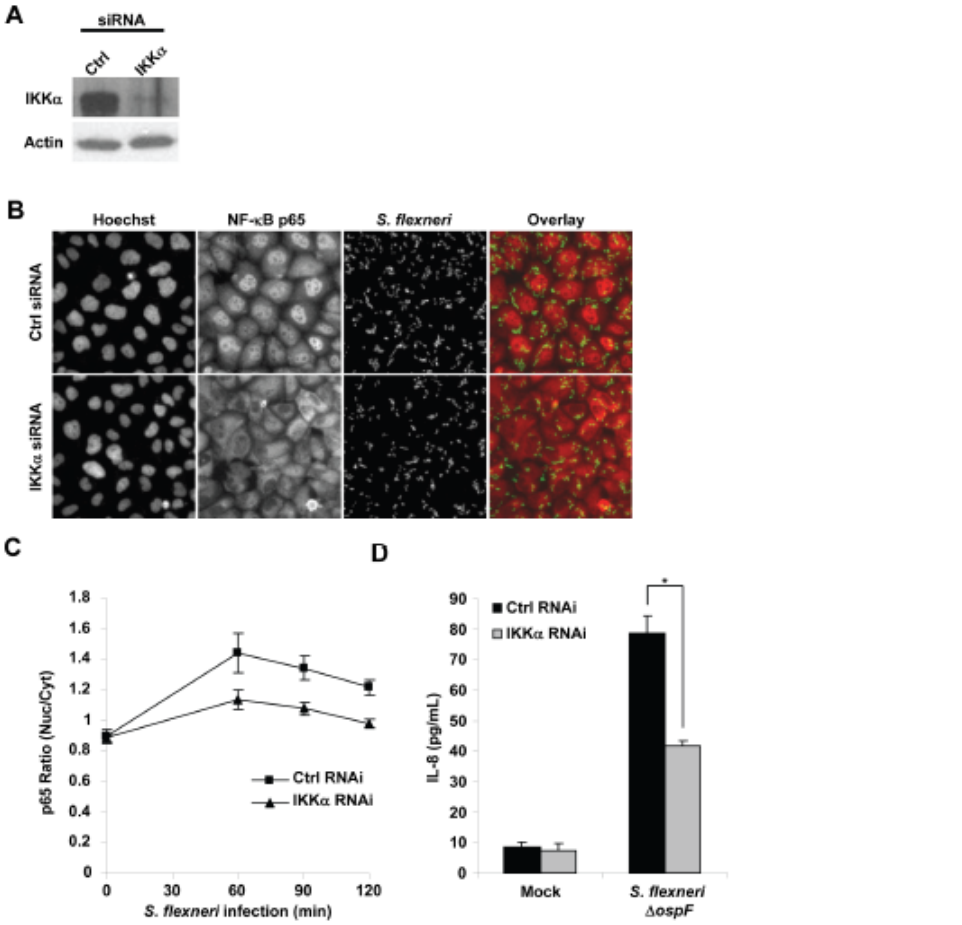

Figure 2. Depletion of IKKα inhibits *S. flexneri*-induced p65 nuclear translocation and IL-8 expression without affecting bacterial invasion. (A) IKKα expression is effectively reduced by RNAi. HeLa cells were transfected with the indicated siRNAs. Western blots were performed on cell lysates. Actin levels are shown as a loading control. (B) Depletion of IKKα inhibits *S. flexneri*-induced p65 nuclear translocation and IL-8 expression without affecting bacterial invasion. HeLa cells were transfected with control or IKKα siRNA and infected with *S. flexneri* for 60 minutes at MOI = 10. An overlay is shown for *S. flexneri* (green) and p65 (red), right panel. (C) Time course of p65 nuclear translocation during *S. flexneri* infection of control and IKKα-depleted cells. Results represent the mean ± SD of 12 images; graph representative of 2 independent experiments. (D) *S. flexneri*-induced IL-8 secretion is impaired by the depletion of IKKα. IL-8 secretion was measured by ELISA in the supernatant of control or IKKα-depleted HeLa cells left untreated (Mock) or infected with *S. flexneri* Δ*ospF*. Results represent the mean ± SD of 6 wells; graph representative of 3 independent experiments, *p = 0.006.

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Depletion of IKKα inhibits *S. flexneri*-induced p65 nuclear translocation and IL-8 expression without affecting bacterial invasion

First, to validate the role of IKKα in *S. flexneri*-induced p65 nuclear translocation, we tested a pool of four synthetic IKKα siRNAs that had no overlapping sequences with the *in vitro* diced siRNAs used in the screen. In conditions where around 90% of endogenous protein was depleted (Figure 2A), a massive reduction of p65 nuclear translocation was observed (Figures 2B and 2C). This result was not explained by a reduction of bacterial uptake as the number of internalized bacteria was similar in control and in IKKα-depleted cells (Figure 2B). Taken together, these results indicated that IKKα is required for the activation of NF-κB during infection of epithelial cells by *S. flexneri*.

During infection, NF-κB positively regulates the expression of multiple proinflammatory genes [31]. In particular, it induces the expression of the chemokine IL-8, which recruits PMNs on site of infection, and thereby limits the spread of bacterial invasion within the intestinal tissue [18]. To test whether IKKα contributed to the upregulation of IL-8 expression during infection, we measured by ELISA the secretion of IL-8 in the supernatant of control and IKKα-depleted HeLa cells six hours post infection. To increase the amount of IL-8 produced in response to infection, cells were infected with a mutant of *S. flexneri* deleted for the type III effector OspF (*ΔospF*) that dampens inflammation signaling by dephosphorylating p38 in the nucleus of infected cells [20]. Consistent with NF-κB data, a reduction of IL-8 secretion was observed in response to *S. flexneri ΔospF* infection when cells were depleted for IKKα (Figure 2D), showing that IKKα is involved in the signaling pathways that control the expression of a critical inflammatory chemokine during bacterial infection.

Both IKKα and IKKβ contribute to the phosphorylation and the degradation of IκBα during *S. flexneri* infection

It is generally believed that IKKβ and NEMO are the two subunits of the IKK complex involved in the canonical pathway of NF-κB activation. To test the contribution of all IKK subunits in the activation of NF-κB during infection of epithelial cells by *S. flexneri*, we monitored the localization of p65 in cells depleted for IKKα, IKKβ or NEMO. As shown in Figure 3A, the depletion of either of these proteins reduced the nuclear translocation of p65, indicating that both catalytic subunits IKKα and IKKβ, as well as the scaffolding function of NEMO were required to fully activate the NF-κB pathway during infection. IKKβ regulates the canonical pathway of NF-κB activation by phosphorylating IκBα at positions serine 32 and 36, thereby inducing its polyubiquitination and subsequent degradation [9]. To test whether IKKα regulated NF-κB via a similar mechanism, we monitored the phosphorylation of IκBα at serines 32 and 36 (pIκBα) and its degradation following infection of HeLa cells by *S. flexneri*. Whereas massive phosphorylation and degradation of IκBα were observed in control cells following infection, these two processes were strongly reduced after IKKα knockdown (Figure 3B), indicating that IKKα largely contributes to the phosphorylation and degradation of IκBα during infection by *S. flexneri*.

IKKα is involved in Nod1-mediated peptidoglycan recognition

During *S. flexneri* infection, the activation of NF-κB is initiated by the recognition of peptidoglycan fragments via the intracellular receptor Nod1 [16]. To specifically examine the implication of IKKα in Nod1-mediated signaling, the effect of IKKα depletion was directly tested in cells exposed to the Nod1 ligand Tri-DAP. Whereas Tri-DAP treatment induced a clear nuclear translocation of p65 in control cells, this translocation was severely impaired in IKKα-depleted cells (Figures 4A and 4B), demonstrating that IKKα is required for the activation of NF-κB following Nod1-mediated peptidoglycan recognition.

Discussion

Although NF-κB is a transcription factor that has been the subject of intensive research by academic laboratories and the pharmaceutical industry, the complex molecular mechanisms controlling its activation are only partially elucidated. Here we performed an RNAi screen to identify new proteins involved in the mechanisms that control NF-κB activation following pathogen recognition using infection of epithelial cells by the invasive

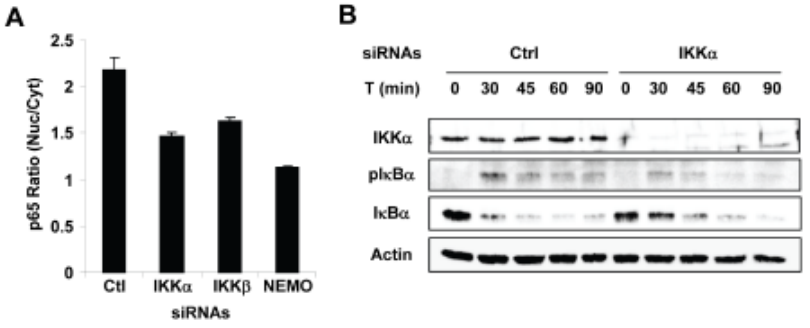

Figure 3. Both IKKα and IKKβ catalytic subunits contribute to NF-κB activation during infection by *S. flexneri*. (A) Depletion of IKKα, IKKβ or NEMO reduces the translocation of p65 during *S. flexneri*. *S. flexneri*-induced p65 translocation was analyzed in HeLa cells transfected with control, IKKα, IKKβ and NEMO siRNAs. Results represent the mean ± SD of 12 images; graph representative of 2 independent experiments. (B) IKKα is involved in the phosphorylation and the degradation of IκBα during *S. flexneri* infection. Control or IKKα-depleted HeLa cells were left untreated or infected with *S. flexneri* for the indicated periods at MOI = 10. Levels of IKKα, pIκBα and IκBα were monitored by western blots performed on cell lysates. Actin levels are shown as a loading control.
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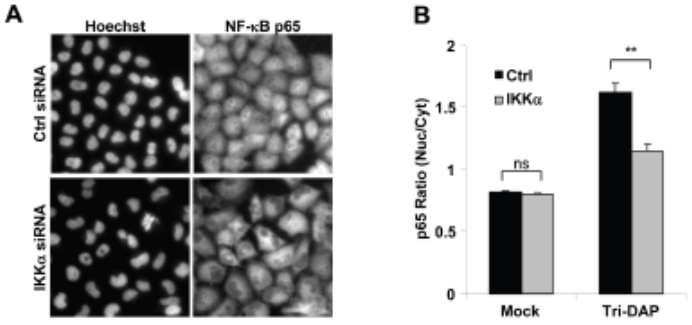

Figure 4. IKKα is required for the activation of NF-κB induced by Nod1-mediated peptidoglycan recognition. (A) IKKα depletion reduces the activation of NF-κB induced by Nod1-mediated peptidoglycan recognition. Control or IKKα-depleted HeLa cells were treated with Tri-DAP as described in Materials and Methods. After 1 hour, cells were fixed, stained for p65 and DNA with an anti-p65 antibody and Hoechst, respectively. (B) Quantification of the effect of IKKα depletion on Tri-DAP-induced p65 nuclear translocation. Results represent the mean ± SD of 12 images; graph representative of 3 independent experiments, **p≤8.6E-4, ns: non-significant.
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bacterium *S. flexneri* as a model system. The presence of *S. flexneri* in the cytoplasm is recognized via the detection of peptidoglycan by the receptor Nod1 [16]. This recognition leads to NF-κB activation and the upregulation of proinflammatory genes that orchestrate the host inflammatory response [31]. The protein IKKα was identified in the screen as a protein required for the activation of NF-κB in response to *S. flexneri* infection. Since bacterial uptake was not affected by the depletion of IKKα, this result indicated that IKKα is involved in the activation of NF-κB in response to infection. The depletion of IKKβ or NEMO also induced a reduction of *S. flexneri*-induced-p65 nuclear translocation, confirming that all subunits were required for the full activation of NF-κB during infection. As the translocation of p65 depends on the degradation of IκBα primed by its phosphorylation on serines 32 and 36, the role of IKKα in these two processes was analyzed. A reduction of IκBα phosphorylation and delayed degradation were observed in cells depleted for IKKα, indicating that similar to IKKβ, IKKα triggers NF-κB activation by inducing the phosphorylation and the degradation of IκBα. Taken together, these results provided new evidence for a role of IKKα in the canonical pathway of NF-κB activation, and were in line with few reports indicating that the dominant model of canonical NF-κB activation based on IKKβ and NEMO is incomplete. In particular, it has been reported that IKKα is the key subunit responsible for the Receptor activator of NF-κB (RANK)-induced classical NF-κB activation in mammary epithelial cells [32]. In addition, Solt et al. showed that IL-1-induced NF-κB activation requires the interaction of IKKα with NEMO and occurs in the absence of IKKβ [11]. Finally, it has been shown recently that both IKKα and IKKβ contribute to IκBα phosphorylation and NF-κB activation in response to TNFα stimulation in HeLa cells [10]. For the first time, our results demonstrate that IKKα is also implicated in the canonical pathway of NF-κB activation triggered by bacterial infection. To further demonstrate that pathogen recognition induced NF-κB activation in an IKKα-dependent manner during *S. flexneri* infection, we directly tested whether IKKα was involved in the activation of NF-κB induced by Nod1-mediated peptidoglycan recognition. For this purpose, controls or IKKα-depleted cells were directly stimulated with the purified Nod1 ligand Tri-DAP. The analysis of the p65 nuclear translocation showed that IKKα was required for the full activation of NF-κB in response to Tri-DAP treatment, indicating that IKKα is involved in the molecular mechanism signaling the recognition of peptidoglycan-derived peptides by Nod1.

Following infection of epithelial cells by *S. flexneri*, the activation of NF-κB leads to the upregulation of genes encoding for inflammatory cytokines including IL-8 and TNFα [31]. To confirm the implication of IKKα in the expression of genes induced by the canonical NF-κB pathway, the deletion of IKKα was tested on *S. flexneri*-induced IL-8 expression. Consistent with NF-κB data, the secretion of IL-8 was reduced when IKKα was depleted, showing that the contribution of IKKα to NF-κB activation has a functional impact on the amplitude of the inflammatory response mounted in response to bacterial infection. These results suggest that inhibition of IKKα activity may be critical to control inflammation upon bacterial infection. Inhibitors of IKKβ have demonstrated therapeutic benefits in various animal models of inflammatory diseases and are currently in early clinical trials [33,34,35]. The data presented in this manuscript suggest that IKKα inhibitors should also be developed and used in combination with IKKβ inhibitors to limit inflammation during bacterial infection or in inflammatory disorders that may involve Nod1 signaling, including asthma, eczema and inflammatory bowel diseases [36,37,38].

Supporting Information

Table S1 Results of the screen on *S. flexneri*-induced p65 nuclear translocation. Scores are fold standard deviations from the mean of GL3 control p65 ratios. Gene names are based on the NCBI nomenclature. (PDF)

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Author Contributions

Conceived and designed the experiments: MLK CA. Performed the experiments: MLK HGJ. Analyzed the data: MLK CA. Contributed reagents/materials/analysis tools: CAK. Wrote the paper: CA.

References

1. Medzhitov R (2007) Recognition of microorganisms and activation of the immune response. Nature 449: 819–826.
2. Takeuchi O, Akira S Pattern recognition receptors and inflammation. Cell 140: 805–820.
3. Blasius AL, Beutler B Intracellular toll-like receptors. Immunity 32: 305–315.
4. Franchi L, Park JH, Shaw MH, Marina-Garcia N, Chen G, et al. (2008) Intracellular NOD-like receptors in innate immunity, infection and disease. Cell Microbiol 10: 1–8.
5. Kawai T, Akira S (2008) Toll-like receptor and RIG-I-like receptor signaling. Ann N Y Acad Sci 1143: 1–20.
6. Rasmussen SB, Reinert LS, Paludan SR (2009) Innate recognition of intracellular pathogens: detection and activation of the first line of defense. Apmis 117: 323–337.
7. Beutler B (2009) Microbe sensing, positive feedback loops, and the pathogenesis of inflammatory diseases. Immunol Rev 227: 248–263.
8. Hayden MS, Ghosh S (2004) Signaling to NF-kappaB. Genes Dev 18: 2195–2224.
9. Solt LA, May MJ (2008) The IkappaB kinase complex: master regulator of NF-kappaB signaling. Immunol Res 42: 3–18.
10. Adli M, Merkhofer E, Cogswell P, Baldwin AS IKKalpha and IKKbeta each function to regulate NF-kappaB activation in the TNF-induced/canonical pathway. PLoS One 5: e9428.
11. Solt LA, Madge LA, Orange JS, May MJ (2007) Interleukin-1-induced NF-kappaB activation is NEMO-dependent but does not require IKKbeta. J Biol Chem 282: 8724–8733.
12. Gilmore TD (2006) Introduction to NF-kappaB: players, pathways, perspectives. Oncogene 25: 6680–6684.
13. Hayden MS, Ghosh S (2008) Shared principles in NF-kappaB signaling. Cell 132: 344–362.
14. Schroeder GN, Hilbi H (2008) Molecular pathogenesis of Shigella spp.: controlling host cell signaling, invasion, and death by type III secretion. Clin Microbiol Rev 21: 134–156.
15. Islam D, Veress B, Bardhan PK, Lindberg AA, Christensson B (1997) In situ characterization of inflammatory responses in the rectal mucosae of patients with shigellosis. Infect Immun 65: 739–749.
16. Girardin SE, Boneca IG, Carneiro LA, Antignac A, Jehanno M, et al. (2003) Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan. Science 300: 1584–1587.
17. Inohara N, Koseki T, Lin J, del Peso L, Lucas PC, et al. (2000) An induced proximity model for NF-kappa B activation in the Nod1/RICK and RIP signaling pathways. J Biol Chem 275: 27823–27831.
18. Sansonetti PJ, Arondel J, Huerre M, Harada A, Matsushima K (1999) Interleukin-8 controls bacterial transepithelial translocation at the cost of epithelial destruction in experimental shigellosis. Infect Immun 67: 1471–1480.
19. Ogawa M, Handa Y, Ashida H, Suzuki M, Sasakawa C (2008) The versatility of Shigella effectors. Nat Rev Microbiol 6: 11–16.
20. Arbibe L, Kim DW, Batsche E, Pedron T, Mateescu B, et al. (2007) An injected bacterial effector targets chromatin access for transcription factor NF-kappaB to alter transcription of host genes involved in immune responses. Nat Immunol 8: 47–56.
21. Li H, Xu H, Zhou Y, Zhang J, Long C, et al. (2007) The phosphothreonine lyase activity of a bacterial type III effector family. Science 315: 1000–1003.
22. Brandman O, Liou J, Park WS, Meyer T (2007) STIM2 is a feedback regulator that stabilizes basal cytosolic and endoplasmic reticulum Ca2+ levels. Cell 131: 1327–1339.
23. Galvez T, Teruel MN, Heo WD, Jones JT, Kim ML, et al. (2007) siRNA screen of the human signaling proteome identifies the Pdlim(3,4,5)P3-mTOR signaling pathway as a primary regulator of transferrin uptake. Genome Biol 8: R142.
24. Liou J, Kim ML, Heo WD, Jones JT, Myers JW, et al. (2005) STIM is a Ca2+ sensor essential for Ca2+-store-depletion-triggered Ca2+ influx. Curr Biol 15: 1235–1241.
25. Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proc Natl Acad Sci U S A 97: 6640–6645.
26. Murphy KC, Campellone KG (2003) Lambda Red-mediated recombinogenic engineering of enterohemorrhagic and enteropathogenic E. coli. BMC Mol Biol 4: 11.
27. Pelkmans L, Fava E, Grabner H, Hannus M, Habermann B, et al. (2005) Genome-wide analysis of human kinases in clathrin- and caveolae/raft-mediated endocytosis. Nature 436: 78–86.
28. Zhang EE, Liu AC, Hirota T, Miraglia LJ, Welch G, et al. (2009) A genome-wide RNAi screen for modifiers of the circadian clock in human cells. Cell 139: 199–210.
29. Girardin SE, Tournebize R, Mavris M, Page AL, Li X, et al. (2001) CARD4/Nod1 mediates NF-kappaB and JNK activation by invasive Shigella flexneri. EMBO Rep 2: 736–742.
30. Dehio C, Prevost MC, Sansonetti PJ (1995) Invasion of epithelial cells by Shigella flexneri induces tyrosine phosphorylation of cortactin by a pp60c-src-mediated signalling pathway. Embo J 14: 2471–2482.
31. Pedron T, Thibault C, Sansonetti PJ (2003) The invasive phenotype of Shigella flexneri directs a distinct gene expression pattern in the human intestinal epithelial cell line Caco-2. J Biol Chem 278: 33878–33886.
32. Cao Y, Bonizzi G, Seagroves TN, Greten FR, Johnson R, et al. (2001) IKKalpha provides an essential link between RANK signaling and cyclin D1 expression during mammary gland development. Cell 107: 763–775.
33. Ismailova ES, Paz N, Alencar H, Chen M, Schopf L, et al. (2007) Use of molecular imaging to quantify response to IKK-2 inhibitor treatment in murine arthritis. Arthritis Rheum 56: 117–128.
34. Ogawa H, Azuma M, Mato S, Nishioka Y, Honjo A, et al. IkappaB kinase beta inhibitor IMD-0354 suppresses airway remodelling in a Dermatophagoides pteronyssinus-sensitized mouse model of chronic asthma. Clin Exp Allergy.
35. Ziegelbauer K, Gantner F, Lukacs NW, Berlin A, Fuchikami K, et al. (2005) A selective novel low-molecular-weight inhibitor of IkappaB kinase-beta (IKK-beta) prevents pulmonary inflammation and shows broad anti-inflammatory activity. Br J Pharmacol 145: 178–192.
36. Le Bourhis L, Benko S, Girardin SE (2007) Nod1 and Nod2 in innate immunity and human inflammatory disorders. Biochem Soc Trans 35: 1479–1484.
37. Reijmerink NE, Bottema RW, Kerkhof M, Gerritsen J, Stelma FF, et al. TLR-related pathway analysis: novel gene-gene interactions in the development of asthma and atopy. Allergy 65: 199–207.
38. Weidinger S, Klopp N, Rummler L, Wagenpfeil S, Novak N, et al. (2005) Association of NOD1 polymorphisms with atopic eczema and related phenotypes. J Allergy Clin Immunol 116: 177–184.

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PRAS40 and PRR5-Like Protein Are New mTOR Interactors that Regulate Apoptosis

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TOR (Target of Rapamycin) is a highly conserved protein kinase and a central controller of cell growth. TOR is found in two functionally and structurally distinct multiprotein complexes termed TOR complex 1 (TORC1) and TOR complex 2 (TORC2). In the present study, we developed a two-dimensional liquid chromatography tandem mass spectrometry (2D LC-MS/MS) based proteomic strategy to identify new mammalian TOR (mTOR) binding proteins. We report the identification of Proline-rich Akt substrate (PRAS40) and the hypothetical protein Q6MZQ0/FLJ14213/CAE45978 as new mTOR binding proteins. PRAS40 binds mTORC1 via Raptor, and is an mTOR phosphorylation substrate. PRAS40 inhibits mTORC1 autophosphorylation and mTORC1 kinase activity toward eIF-4E binding protein (4E-BP) and PRAS40 itself. HeLa cells in which PRAS40 was knocked down were protected against induction of apoptosis by TNFα and cycloheximide. Rapamycin failed to mimic the pro-apoptotic effect of PRAS40, suggesting that PRAS40 mediates apoptosis independently of its inhibitory effect on mTORC1. Q6MZQ0 is structurally similar to proline rich protein 5 (PRR5) and was therefore named PRR5-Like (PRR5L). PRR5L binds specifically to mTORC2, via Rictor and/or SIN1. Unlike other mTORC2 members, PRR5L is not required for mTORC2 integrity or kinase activity, but dissociates from mTORC2 upon knock down of tuberous sclerosis complex 1 (TSC1) and TSC2. Hyperactivation of mTOR by TSC1/2 knock down enhanced apoptosis whereas PRR5L knock down reduced apoptosis. PRR5L knock down reduced apoptosis also in mTORC2 deficient cells. The above suggests that mTORC2-dissociated PRR5L may promote apoptosis when mTOR is hyperactive. Thus, PRAS40 and PRR5L are novel mTOR-associated proteins that control the balance between cell growth and cell death.

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INTRODUCTION

TOR (Target of Rapamycin) kinase is a highly conserved, central controller of cell growth [1–3]. The fundamental importance of TOR is underscored by genetic studies showing TOR to be essential for cell growth and development; disruption of the *TOR* gene is lethal in all examined species [4–12]. In humans, dysfunctional mTOR signaling plays an important role in many if not most cancers, as well as in diseases such as tuberous sclerosis complex (TSC, #191100 OMIM) and lymphangiomyelomatosis (LAM, #606690 OMIM). TOR is found, from yeast to human, in two functionally and structurally distinct multiprotein complexes termed TOR complex 1 (TORC1) and TORC2 [13–15]. The rapamycin-sensitive mammalian TOR complex 1 (mTORC1) consists of mTOR, mLST8 and Raptor [13,16,17]. mTORC2 contains Rictor and SIN1 instead of Raptor, and is rapamycin-insensitive [14,15,18–20]. Knock out of Raptor, SIN1 or Rictor in mice is embryonic lethal, indicating that both mTORC1 and mTORC2 are essential [19–22].

mTORC1 is activated by nutrients (amino acids), anabolic growth factors (e.g., insulin and insulin-like growth factor), and cellular energy (ATP) [1–3]. The growth factor signal and energy status are transmitted to mTORC1 via the essential tumor suppressor tuberous sclerosis complex (TSC) proteins TSC1 and TSC2 [23,24]. The TSC heterodimer (TSC1-TSC2) is a GTPase activating protein (GAP) that inhibits the essential small GTPase Rheb [25,26]. Rheb-GTP binds and activates mTORC1 [27]. Akt (also known as PKB) phosphorylates and inactivates TSC2 in response to growth factors [28], whereas AMPK phosphorylates and activates TSC2 in response to low energy (high AMP) [29,30]. Nutrients impinge on mTORC1 at the level of Rheb or mTORC1 by a poorly understood mechanism involving the type III PI3K hVps34 [27,31]. The upstream regulators of the more recently identified mTORC2 are not known, but mTORC2 appears to respond at least to growth factors, possibly via TSC1-TSC2 [32].

mTORC1 and mTORC2 separately control many cellular processes that collectively determine cell growth and development. mTORC1 controls transcription, protein synthesis, ribosome biogenesis, nutrient transport, and autophagy, among other processes. mTORC1 controls protein synthesis via phosphorylation of S6 kinase (S6K) and eIF-4E binding protein (4E-BP), two key regulators of translation initiation [3,33,34]. mTORC2 controls organization of the actin cytoskeleton via small Rho-type GTPases and Protein Kinase C [14,15,35], and thereby determines the shape and possibly motility of the cell. In addition, mTORC2 phosphorylates Ser473 in the hydrophobic motif of Akt and thereby activates Akt toward substrates such as the Forkhead transcription factor FOXO and the apoptosis regulator BAD [19–21,36].

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☯ These authors contributed equally to this work.

Although upstream regulators of mTOR, at least for mTORC1, are relatively well characterized, astonishingly few direct substrates and downstream effectors of the mTORCs are known. This is particularly true for mTORC2 which was discovered only recently and, due to its rapamycin insensitivity, is not pharmacologically addressable. To identify additional regulators, substrates, and/or components of the mTORCs, we developed a highly sensitive mass spectrometry-based screen. Here we report the identification of two novel mTOR binding proteins, PRAS40 (Q96B36 Swiss-Prot) and PRR5L (Q6MZQ0 Swiss-Prot), which bind specifically to mTORC1 and mTORC2, respectively. We further characterize the roles of these two proteins in mTOR complex formation and function.

RESULTS AND DISCUSSION

PRAS40 and PRR5L bind specifically to mTORC1 and mTORC2

To identify new mTOR binding proteins, we used a 'gel-less' mass spectrometry-based method to screen for mTOR associated proteins. mTOR complexes, first purified by large scale immunoprecipitations (IPs) with antibody directed against mTOR, were digested with trypsin and after detergent removal subjected directly to two-dimensional liquid chromatography tandem mass spectrometry (2D LC-MS/MS). Within a single 2D LC-MS/MS run up to 270 different proteins were identified. To identify specific mTOR interactors, we compared mTOR and mock IPs, and chose those proteins that were present only in the mTOR IPs. Furthermore, to qualify as a specific interactor, a protein had to be identified in at least three out of four independent mTOR IP experiments. We reproducibly identified all known members of the mTOR complexes (Table 1). The sequence coverage for mTOR, Rictor, mLST8 and Raptor was about 20%, while sequence coverage for SIN1 was 13.8%. The above experiment was repeated with antibodies specific for Rictor or Raptor and, as expected, mTOR and mLST8 were found in both the Rictor and Raptor IPs, whereas SIN1 was found only in the Rictor IP.

In addition to the known mTORC partners, we also identified novel mTOR interacting proteins. The proline-rich Akt substrate PRAS40 (10.6% sequence coverage) was found in mTOR and Raptor IPs but not in Rictor IPs (Table 1). These interactions were confirmed by co-IP experiments with HeLa and HEK293 cells (Figure 1). PRAS40 is therefore a specific mTORC1 binding partner (Figure 1A). PRAS40 was originally discovered as an Akt substrate of unknown function [37]. During the preparation of this manuscript, two studies appeared suggesting that PRAS40 is an mTORC1 inhibitor [38,39]. In addition to PRAS40, we identified the hypothetical protein Q6MZQ0/FLJ14213/CAE45978 as a specific mTORC2 interactor (Table 1). Q6MZQ0 was cloned with an N-terminal GST tag and its interaction with mTORC2 was confirmed by co-IP and GST pull downs from HeLa and HEK293 cells (Figure 1B). Since Q6MZQ0 displays 39% sequence similarity with the proline rich protein PRR5, we named it PRR5-Like protein (PRR5L). PRR5L is an uncharacterized protein. The related protein PRR5, however, is highly expressed in kidney and has been suggested to be a tumor suppressor since it is down regulated in a subset of breast tumors [40].

Earlier studies on the mTORCs failed to detect PRAS40 and PRR5L possibly because both have an apparent molecular weight of approximately 40 kDa as measured by SDS-PAGE. Former searches for mTOR binding proteins relied on IPs followed by SDS-PAGE analysis. The co-migrating heavy chain of the IP antibody used in these earlier experiments might have masked PRAS40 and PRR5L. Our 2D LC-MS/MS approach also identified Transferrin Receptor 1 (P02786 Swiss-Prot), NICE-4 (Q14157 Swiss-Prot), Plectin 1 (Q6S383 Swiss-Prot), and Thymopoietin (P42166 Swiss-Prot) as potential mTOR binding proteins, but direct co-IP experiments indicated that these were non-specific binding proteins (data not shown).

PRAS40 binds mTORC1 via Raptor

Following rapamycin treatment, PRAS40 dissociated from mTOR (Figure 1A). However, released PRAS40 remained bound to Raptor which, as reported previously [41], is also released from mTOR upon rapamycin treatment. Furthermore, PRAS40 binding to mTOR was strongly reduced when Raptor was knocked down (Figure 2A). These findings indicate that PRAS40 binds mTORC1 via Raptor. We also observed that PRAS40 associated less well with a kinase dead version of mTOR (Figure 2B), suggesting that mTORC1-mediated phosphorylation of PRAS40 (see below) may affect the PRAS40- mTORC1 interaction. Our findings are in agreement with recent PRAS40 studies showing that PRAS40 binds preferentially to Raptor [38], and that the mTOR kinase domain is also involved in PRAS40 binding [39].

Table 1. mTORC1 and mTORC2 associated proteins identified by 2D-LC-MS/MS

Protein	# Identifications (out of 4 independent IPs)	Sequence Coverage	Identified in IP of			Predicted MW (Da)	Length (aa)
			mTOR	Rictor	Raptor		
mTOR	4	26.2%	x	x	x	288892	2549
Rictor	4	21%	x	x		192217	1708
Raptor	4	18.2%	x		x	149038	1335
SIN1	4	13.8%	x	x		59123	522
PRR5L	3	5.2%	x	x		40866	368
mLST8	4	21.5%	x	x	x	35902	326
PRAS40	3	10.6%	x		x	27383	256

mTOR complexes were purified by immunoprecipitation (IP) with antibody directed against mTOR, Rictor or Raptor. Immunoprecipitates were analyzed by 2D LC-MS/MS. Proteins that were found in at least three out of four mTOR IPs, but not in mock IPs, were considered specific. IPs with antibodies directed against Rictor or Raptor indicated whether a candidate was specific for mTORC1 or mTORC2, respectively.

doi:10.1371/journal.pone.0001217.t001

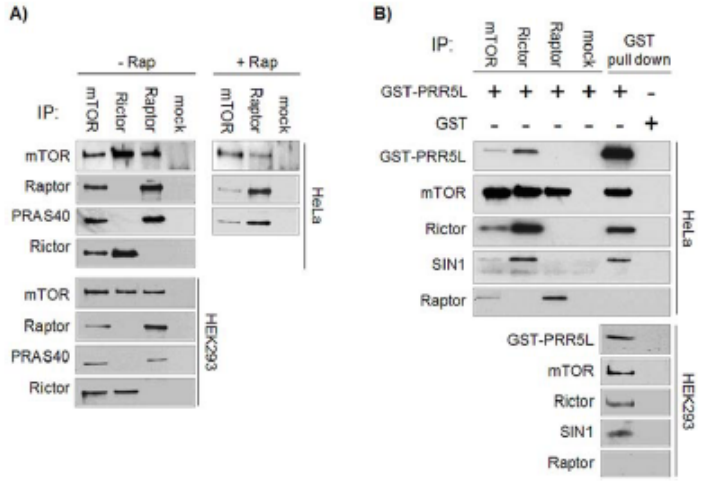

Figure 1. Confirmation of PRAS40 and PRR5L binding to mTOR. A. PRAS40 is associated specifically with mTORC1. mTOR, Rictor, Raptor and mock IPs were performed with HeLa and HEK293 extracts and analyzed by immunoblotting. PRAS40 was found specifically in mTOR and Raptor IPs. 1 h rapamycin treatment of cells dissociated a Raptor-PRAS40 subcomplex from mTOR **B. PRR5L is associated specifically with mTORC2.** HeLa or HEK293 cells were transfected with GST-PRR5L or the empty plasmid. mTOR, Rictor, Raptor and mock IPs and GST pull downs were analyzed by immunoblotting. GST-PRR5L is detected specifically in mTOR and Rictor IPs. mTOR, Rictor and SIN1, but not Raptor, are detected specifically in GST-PRR5L pull downs.
doi:10.1371/journal.pone.0001217.g001

PRAS40 is a substrate and an inhibitor of mTORC1 *in vitro*

The above results suggested that PRAS40 is phosphorylated by mTORC1. To investigate if PRAS40 is an mTORC1 substrate, we performed *in vitro* kinase assays with mTORC1 or mTORC2 and purified PRAS40. PRAS40 was phosphorylated weakly by both mTORC1 and mTORC2. Importantly, we also found that PRAS40 inhibited mTORC1 autophosphorylation but not mTORC2 autophosphorylation (Figure 3A), suggesting that the weak phosphorylation of PRAS40 by mTORC1 might be due to PRAS40-mediated inhibition of mTORC1. To investigate whether PRAS40 inhibits mTORC1 kinase activity, we performed *in vitro* kinase assays with the known mTORC1 substrate 4E-BP and increasing concentrations of purified PRAS40. PRAS40 inhibited both mTORC1 autophosphorylation and mTORC1 phosphorylation of 4E-BP, in a concentration dependent manner (Figure 3B). In addition, PRAS40 phosphorylation inversely correlated with the concentration of PRAS40 in the kinase reaction, suggesting that PRAS40 is indeed both a substrate and an inhibitor of mTORC1 kinase activity. Our finding that PRAS40 inhibits mTORC1 kinase activity toward 4E-BP and PRAS40 is in agreement with the observation of Sancak et al. [38] and Vander Haar et al. [39] that PRAS40 inhibits mTORC1 toward S6K1. Hence, we conclude that PRAS40 is a broad mTORC1 inhibitor that inhibits mTORC1 kinase activity toward itself, 4E-BP, S6K1, and PRAS40. It remains to be determined whether mTORC1-mediated phosphorylation of PRAS40 plays a role in PRAS40's ability to bind and inhibit mTORC1.

Interestingly, we found that phosphorylation of the Akt consensus site T246 in PRAS40 is moderately reduced in Rictor knock down cells (data not shown). This suggests that mTORC2 may activate Akt toward PRAS40. This in turn suggests that mTORC2, via PRAS40, may be upstream of mTORC1. If mTORC2 is indeed upstream of mTORC1, it might be only under specific conditions or only with regard to particular mTORC1 substrates (other than S6K1), as we and others failed to detect an effect of mTORC2 disruption on S6K1 phosphorylation [14,15]. The potential regulation of mTORC1 by mTORC2 requires further investigation.

PRAS40 deficiency prevents induction of apoptosis by TNFα and cycloheximide

Constitutively active mTOR reduces apoptosis [42] whereas inhibition of mTORC1 with rapamycin induces or facilitates apoptosis in several cell lines [43–48]. We therefore reasoned that the mTORC1 inhibitor PRAS40 might promote apoptosis and that PRAS40 knock down would thus protect cells against the induction of apoptosis. To investigate this possibility, we examined the effect of PRAS40 knock down on the sensitivity of HeLa cells to apoptosis induction by TNFα in combination with cycloheximide. To monitor apoptosis, treated cells were processed for visualization of DNA and cleaved PARP. We found that apoptosis was reduced in PRAS40 knock down cells (Figure 4A), suggesting that PRAS40 is indeed pro-apoptotic. To analyze if PRAS40 promotes apoptosis via its inhibitory effect on mTORC1, we investigated if rapamycin suppressed the effect of a PRAS40 deficiency on TNFα/cycloheximide induced apoptosis in HeLa cells. Rapamycin failed to prevent the reduction in apoptosis caused by PRAS40 knock down (Figure 4B). In addition, rapamycin treatment did not affect apoptosis induction by TNFα/cycloheximide in control cells (Figure 4B), even after 6h of rapamycin treatment (data not shown). The finding that rapamycin failed to mimic the pro-apoptotic effect of PRAS40, suggests that PRAS40 mediates apoptosis independently of its

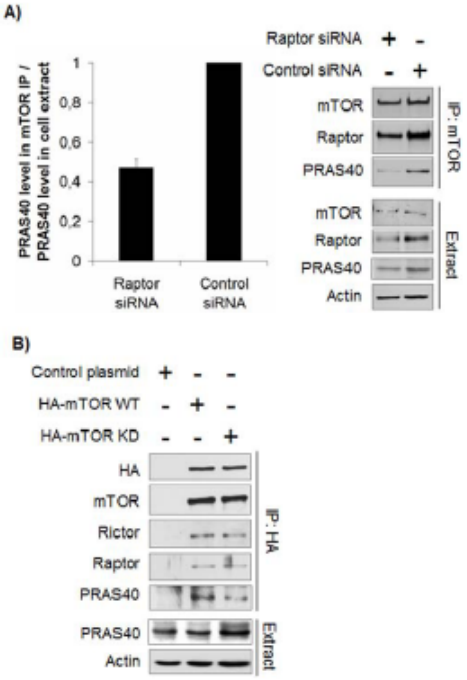

Figure 2. PRAS40 interacts with mTORC1. A. PRAS40 binds mTOR via Raptor. HEK293 cells were transfected with Raptor or control siRNA vectors and incubated for 4 days, followed by mTOR IP and immunoblotting. mTOR IPs and cell extracts were probed with antibodies directed against the indicated proteins. Since knock down of Raptor reduced the cellular amount of PRAS40, the PRAS40 signal in the IPs was quantified relative to PRAS40 levels in the corresponding extract. Quantitations were averaged over three independent experiments. Raptor knock down reduced the amount of PRAS40 associated with mTOR by 50%, as compared to control cells. **B. mTOR kinase domain is involved in PRAS40 binding.** HEK293 cells were transfected with a plasmid expressing wild type (WT) HA-mTOR or kinase dead (KD) HA-mTOR or an empty control plasmid, and incubated for 48 h followed by extract preparation and IP with an anti-HA antibody. PRAS40 levels in the extracts remained unaltered. PRAS40 association with mTORC1 containing HA-mTOR KD was moderately reduced as compared with mTORC1 containing HA-mTOR WT.
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inhibitory effect on mTORC1. However, PRAS40 T246 phosphorylation (PRAS40-pT246) appears to protect neuronal cells from apoptosis after stroke [49]. PRAS40-pT246 has also been proposed to promote cell survival in cancer cells [50]. The fact that T246 is the site via which Akt negatively regulates PRAS40's ability to inhibit mTORC1 [38] suggests that PRAS40 may indeed be pro-apoptotic via its ability to inhibit mTORC1. To explain the apparent discrepancy with our inability to induce apoptosis with rapamycin, PRAS40 may have to inhibit both mTORC1 and a second, unknown rapamycin insensitive target (or only this second target) to perform its pro-apoptotic function. mTORC1 may be the more important target in those cell lines where rapamycin induces or facilitates apoptosis [43–48].

PRR5L binds mTORC2 via Rictor/SIN1

Since we initially identified endogenous PRR5L as an mTORC2 binding protein in HeLa cells, we verified PRR5L expression in HEK293 cells by RT-PCR (Figure 5A). PRR5L is strongly expressed in both HeLa and HEK293 cells. Subsequent experiments were performed with HEK293 cells due to the higher transfection efficiency with these cells. To investigate whether PRR5L binds directly to mTOR or via other mTORC2 members, we examined PRR5L binding to mTOR in SIN1 knock down cells. As reported previously [18,19], we observed a reduction in the amount of Rictor when SIN1 was knocked down (Figure 5B), supporting the earlier suggestion that these two proteins stabilize each other. We found that the amount of mTOR in GST-PRR5L pull downs from the SIN1/Rictor deficient cells was substantially reduced compared to control cells (Figure 5B). This suggests that PRR5L binds to mTOR via Rictor and/or SIN1. To investigate whether PRR5L is required for mTORC2 integrity, we examined the binding of mTOR and SIN1 to Rictor in cells knocked down for PRR5L (Figure 5C). The amount of mTOR and SIN1 bound to Rictor, as measured by co-IP, remained unchanged in PRR5L knock down cells. We then investigated if PRR5L is required for mTORC2 kinase activity. Phosphorylation of Akt S473 and Paxillin Y118 is reduced upon mTORC2 disruption [14,15]. However, in PRR5L deficient cells the levels of Akt-pS473 and Paxillin-pY118 remained unchanged (Figure 5D). The findings that PRRL5 is not required for mTORC2 integrity or for phosphorylation of known mTORC2 targets suggest that PRR5L is not an mTORC2 upstream regulator or an integral component of mTORC2.

PRR5L is phosphorylated by mTOR *in vitro*

To determine whether PRR5L is a phosphorylation substrate for mTOR, we performed *in vitro* kinase assays with mTORC1 or mTORC2 and purified PRR5L. We found that PRR5L is phosphorylated by both mTORC1 and mTORC2, the former but not the latter phosphorylation being sensitive to rapamycin treatment (Figure 3A). Hence, PRR5L might be regulated through mTOR phosphorylation. PRR5L addition did not affect mTORC1 or mTORC2 autophosphorylation (Figure 3A), suggesting that PRR5L does not function as an mTORC2 inhibitor like PRAS40 for mTORC1. The significance of the phosphorylation of PRR5L by mTOR, in particular by mTORC1, remains to be determined. The above results taken together suggest that PRR5L is a downstream effector of mTORC2.

PRR5L dissociates from mTORC2 in TSC1/2 deficient cells

To investigate whether TSC1-TSC2 influences PRR5L binding to mTORC2, we examined GST-PRR5L pull downs from TSC1/2 deficient cells. TSC1/2 deficient cells exhibited reduced amounts of Rictor and mTOR bound to GST-PRR5L (Figure 6A). The above finding suggests that PRR5L dissociates from mTORC2 in cells with hyperactive mTOR signaling. It remains to be determined whether the effect of TSC1/2 knock down on PRR5L binding is via mTORC1 or mTORC2. TSC1/2 knock down hyperactivates mTORC1 [1–3] and possibly also mTORC2 (K.D.M and M.N.H., unpublished).

PRR5L promotes apoptosis

Hyperactive mTOR signaling, in TSC knock out MEFs, enhances induction of apoptosis by FCS starvation or TNFα/cycloheximide [51,52]. In agreement, we found that TSC1/2 knock down in human cells, in which PRR5L dissociates from mTORC2, also

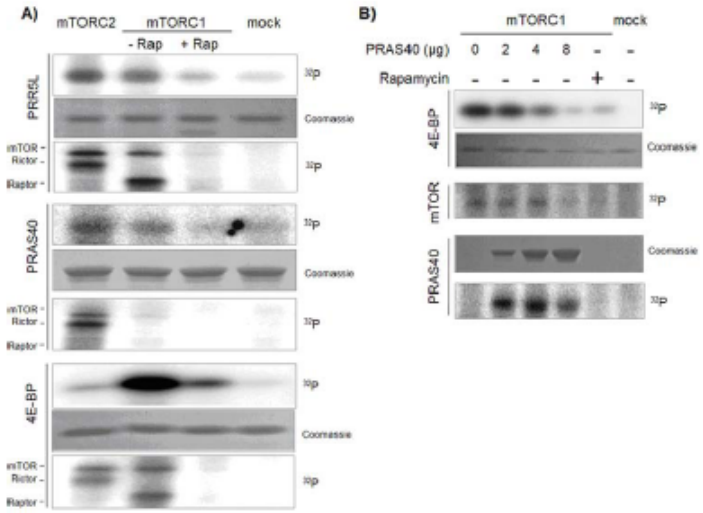

Figure 3. mTOR phosphorylates PRAS40 and PRR5L, and PRAS40 inhibits mTOR kinase activity. A. mTOR phosphorylates PRAS40 and PRR5L. Kinase assays were performed using mTORC1 or mTORC2 immunopurified from HEK293 cells, and purified PRAS40, GST-PRR5L (PRR5L) or 4E-BP as substrates. Rapamycin (100 nM) and purified FKBP12 were added directly to the reaction. Both PRR5L and PRAS40 are phosphorylated *in vitro* by both mTORCs. Phosphorylation by mTORC1 was rapamycin-sensitive. **B. PRAS40 inhibits mTORC1 kinase activity toward 4E-BP and PRAS40 itself.** Kinase assays were performed using mTORC1 immunopurified from HEK293 cells, purified 4E-BP as a substrate, and increasing concentrations of PRAS40. PRAS40 inhibits mTORC1 autophosphorylation and mTORC1 phosphorylation of 4E-BP and PRAS40, in a concentration-dependent manner.
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enhances TNFα/cycloheximide induced apoptosis (Figure 6B). To investigate whether PRR5L plays a role in apoptosis, we examined if PRR5L knock down affects TNFα/cycloheximide induced apoptosis. PRR5L knock down cells were less apoptotic compared to control cells, at various time points after TNFα/cycloheximide treatment, suggesting that PRR5L is pro-apoptotic (Figure 4A and S1). The finding that PRR5L is pro-apoptotic is consistent with the suggested role of the related protein PRR5 as tumor suppressor [40]. Furthermore, the observation that PRR5L is pro-apoptotic and is released from mTORC2 in cells with enhanced apoptosis (TSC1/2 deficient cells) suggests that released PRR5L may promote apoptosis. This in turn suggests that PRR5L is downstream of mTORC2 in mediating apoptosis. To test this possibility, we investigated whether a PRR5L deficiency still reduces apoptosis in cells knocked down for TORC2. We found that cells knocked down for both PRR5L and the mTORC2 component SIN1 were similar to cells knocked down only for PRR5L, with regard to induction of apoptosis by TNFα/cycloheximide (Figure 4C). This observation is consistent with a model in which PRR5L acts downstream of mTORC2. In particular, in response to hyperactive mTOR signaling, PRR5L may dissociate from mTORC2 to promote apoptosis. However, our data do not rule out the possibility that PRR5L controls apoptosis independently of mTORC2. It is important to note that mTORC2 also promotes cell survival via a mechanism other than tethering PRR5L. mTORC2 phosphorylates and activates Akt which then phosphorylates and inactivates the pro-apoptotic factors BAD and FOXO1/3a [19,20,36]. The above taken together suggests that either too much or too little mTOR signaling predisposes a cell to apoptosis. There seems to be a delicate balance between cell growth and cell death that may be mediated at least in part by PRR5L.

In summary, we describe two new mTOR interactors, PRAS40 and PRR5L. PRAS40 binds specifically to mTORC1 whereas PRR5L is mTORC2 specific. PRAS40 binding to mTORC1 is primarily via Raptor but also requires mTOR kinase activity. mTORC1 phosphorylates PRAS40 and this phosphorylation may contribute to the mTORC1-PRAS40 interaction. Furthermore, PRAS40 inhibits mTORC1 autophosphorylation and mTORC1 kinase activity toward its substrates 4E-BP and PRAS40. This observation extends two recent studies showing that PRAS40 inhibits mTORC1 toward its substrate S6K [38,39]. Thus, PRAS40 is an upstream negative regulator of mTORC1. We also show that PRAS40 is pro-apoptotic, but this may be an mTORC1 independent function of PRAS40. PRR5L, the new mTORC2-specific interactor, binds mTOR via SIN1 and/or Rictor. Unlike Rictor and SIN1, PRR5L is not required for mTORC2 integrity or mTORC2 kinase activity toward its downstream readouts Akt and Paxillin. Furthermore, we observed that PRR5L binding to mTORC2 is reduced in TSC1/2 deficient cells. We conclude that PRR5L dissociates from mTORC2 in cells with hyperactive mTOR signaling. We show that a TSC1/2 deficiency enhances TNFα/cycloheximide induced apoptosis. Conversely, knock down of PRR5L prevents apoptosis, even in mTORC2 deficient cells. We suggest that PRR5L is downstream of mTORC2 and is pro-apoptotic. It will be of interest to determine whether PRR5L is a tumor suppressor as suggested for the related protein PRR5 [40].

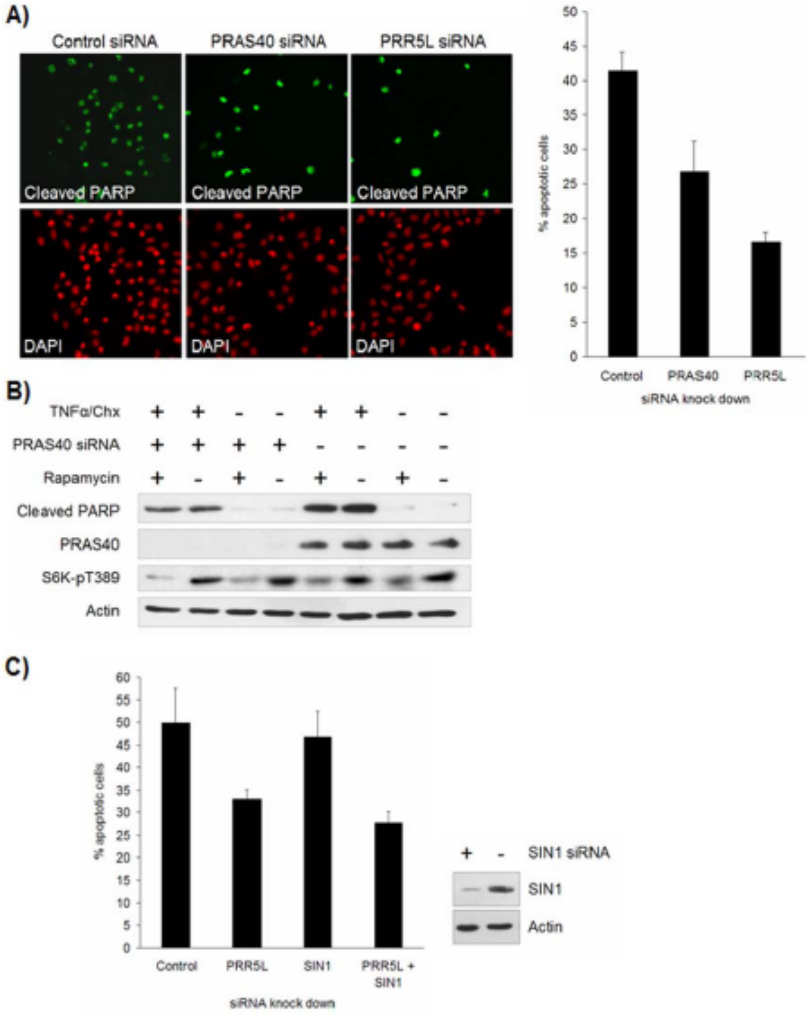

Figure 4. PRAS40 and PRR5L are pro-apoptotic. A. PRR5L and PRAS40 knock down cells are resistant to TNFα/cycloheximide induced apoptosis. HeLa cells were transfected with PRR5L, PRAS40 or control siRNA and incubated for 48 h, followed by 2 h induction of apoptosis with TNFα and cycloheximide. Cells were fixed and stained with DAPI and cleaved PARP antibody, and the percentage of apoptotic cells was quantified. **B. PRAS40's effect on apoptosis is independent of mTORC1.** HeLa cells were transfected with PRAS40 or control siRNA and incubated for 48 h, and treated with 100 nM rapamycin or carrier for 1 h before incubation with TNFα and cycloheximide for 2 h to induce apoptosis. Extracts were analyzed by immunoblotting with the indicated antibodies. **C. PRR5L deficiency protects against apoptosis in SIN1 deficient cells.** HeLa cells were transfected with diced PRR5L siRNA and/or synthetic siRNA against SIN1 as indicated, or the appropriate control siRNAs. Cells were incubated for 48 h, and apoptosis was induced with TNFα and cycloheximide for 2 h. Cells were fixed and stained with DAPI and cleaved PARP antibody, and the percentage of apoptotic cells was quantified. The efficiency of SIN1 knock down was assessed in parallel by immunoblotting (right panel).
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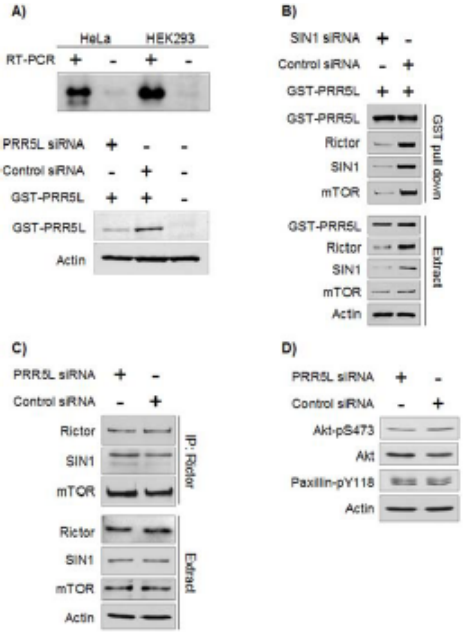

Figure 5. PRR5L binds to mTORC2 via SIN1 and/or Rictor but does not affect mTORC2 integrity or kinase activity. A. PRR5L expression in HeLa and HEK293 cells, and PRR5L knock down efficiency. Top panel: Total RNA was purified from HeLa or HEK293 cells, followed by reverse transcription and PCR with primers corresponding to PRR5L. As a negative control, reverse transcription without the transcriptase enzyme was performed. Endogenous PRR5L is expressed in both cell lines. Bottom panel: HEK293 cells were cotransfected with a GST-PRR5L vector and PRR5L siRNA or control siRNA, and incubated for 48 h. Immunoblots were performed on with antibody against GST or Actin. **B. PRR5L binds mTOR via SIN1 and/or Rictor.** HEK293 cells were cotransfected with a GST-PRR5L vector and a SIN1 siRNA vector or a control siRNA vectors, and incubated for 4 days. GST pull downs were immunoblotted with the indicated antibodies. GST-PRR5L was detected with an anti-GST antibody. mTOR binding to GST-PRR5L is weaker in the absence of SIN1 and Rictor. **C. mTORC2 remains intact in PRR5L knock down cells.** HEK293 cells were transfected with PRR5L siRNA or control siRNA and incubated for 48 h. Rictor IPs were immunoblotted with the indicated antibodies. **D. mTORC2 readouts are unaltered in PRR5L knock down cells.** HEK293 cells were transfected with PRR5L siRNA or control siRNA and incubated for 48 h. Immunoblots were performed on protein extracts with the indicated antibodies. The phosphorylation of Akt S473 and paxillin Y118 is unaltered by PRR5L knock down.
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MATERIALS AND METHODS

Screen for mTOR binding proteins and mass spectrometry

mTOR binding proteins were purified essentially as reported [14]. For each IP experiment, 6 10 cm dishes of HeLa cells at 70% confluence were used. IPs were performed with 6 μg of mTOR (Santa Cruz), Rictor or Raptor (Bethyl), or control goat (Santa Cruz) or rabbit (Bethyl) antibodies. Antibodies were bound to 300 μL magnetic Protein G coupled Dynabeads (Invitrogen). Digestion was performed on the beads [53] with 1 μg of Trypsin (Promega). After drying, detergents were removed by hydrophilic interaction chromatography on PolyHYDROXYETHYL TopTips (PolyLC Inc.) according to the manufacturer's instructions. Ammonium acetate remainders were removed by repeated drying of the samples.

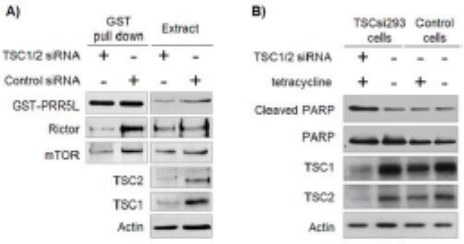

Figure 6. Analysis of TSC1/2 knock down cells. A. PRR5L is released from mTORC2 in TSC1/2 deficient cells. TSC knock down was induced in TSCsi293 cells by tetracycline treatment for 4 days. Cells were cotransfected with GST-PRR5L vector and incubated for 2 days, followed by GST pull downs and immunoblots with the indicated antibodies. GST-PRR5L was detected with an anti-GST antibody. **B. TSC1/2 knock down facilitates apoptosis.** TSCsi293 and T-REx-293 (control) cells were treated with tetracycline for 4 days. Apoptosis was induced by 1.5 h treatment with TNFα and cydoheximide. Extracts were probed with the indicated antibodies.
doi:10.1371/journal.pone.0001217.g006

LC-MS/MS

The peptides were analyzed by two-dimensional capillary liquid chromatography and tandem MS using a PolySULFOETHYL A ion-exchange column (0.15×50 mm, PolyLC, Columbia, MD), connected in series to a C18 trap column (Zorbax 300SB, 0.3×50 mm, Agilent Technologies, Basel, Switzerland), and to a Magic C18 separation column (0.1×100 mm, Thermo Scientific, Basel, Switzerland). The peptides were injected first onto the cation exchange column. Unadsorbed peptides were trapped on the Zorbax column and eluted onto the separation column with a linear 75 min gradient from 2 to 75% B (0.1% acetic acid in 80% acetonitrile) in solvent A (0.1% acetic acid in 2% acetonitrile). Next, peptides that had been retained by the ion-exchange column were sequentially eluted and trapped on the C18 trap column with 10 mL pulses of 50, 100, 150, 200, 250, 300, 350, 400, and 500 mM ammonium acetate, pH 3.3. Peptides eluted by each individual salt pulse were separated by the acetonitrile gradient as described above. The flow was delivered with a Rheos 2200 HPLC system (Thermo Scientific, Basel, Switzerland) at 50 mL/min. A precolumn splitter reduced the flow to approximately 500 nl/min. The eluting peptides were ionized by a Finnigan nanospray ionization source (Thermo Scientific, Basel, Switzerland). The LTQ orbitrap instrument was operated in the data-dependent mode. A survey scan was performed in the Orbitrap between m/z 400-1600 Da at 60,000 resolution. The three most abundant ions detected were fragmented in the LTQ mass spectrometer and mass analyzed in the Orbitrap at a resolution of 7,500. Singly charged ions were not subjected to fragmentation. The normalized collision energy was set to 35%. Individual MS/MS spectra were searched against the NCBI non-redundant databank using the TurboSequest software [54]. The Sequest filter parameters were as follows: Xcorr versus charge state was 1.50 for singly, 2.00 for doubly, and 2.50 for triply charged ions, respectively; the ΔCN was 0.1, and the protein probability was set to 0.01.

Plasmids and reagents

Rictor, Raptor and SIN1 siRNA constructs were previously described [14,20]. An empty pSuper-GFP-neo construct was used as a control. HA-mTOR and kinase dead HA-mTOR constructs were a kind gift from Dr. G. Thomas and were described previously [29], the control empty vector was created by cutting out the mTOR fragment using NotI and PstI.

The coding region of PRR5L was cloned from human cDNA made from HeLa cells, using the following primers: 5′ ATG ACC CGC GGC TTC G 3′ (forward, contained also restriction sites for either BamHI or SpeI), and 5′ T CAG CTG AGG GAA GCA CAG 3′ (reverse, contained also a NotI restriction site). The PCR product was digested either with BamHI and NotI or with SpeI and NotI, and cloned into pGEX-6P-1 or pEBG, respectively. Recombinant GST-PRR5L was expressed from pGEX-6P-1 and purified from *E. coli*.

The generation of the inducible TSC knock down cell line TSCsi293 from an HEK293 cell line (T-REx-293, Invitrogen), that expresses the tetracycline repressor protein TetR, and its handling were as described (K. D. M. and M. N. H., submitted). Cycloheximide was dissolved in water and used at a final concentration of 2.5 μg/mL (Calbiochem), rapamycin was dissolved in DMSO and used at a final concentration of 100 nM (LC laboratories), TNFα was dissolved in PBS containing 0.1% BSA, and used at a final concentration of 10 ng/mL (R&D systems). Purified PRAS40 was from Biosource, purified 4E-BP was from Stratagene.

RNA interference

siRNAs against PRAS40 and PRR5L and control siRNA against Luciferase were generated as described [55] using the following reagents: 5× Megascript T7 Kit (Ambion); Turbo Dicer siRNA Generation Kit (Genlantis); RNA Purification System (Invitrogen). Primers for PRAS40 were as follows: gene specific primers: forward: ttgcctccacgacatcgcac, reverse: tatttccgcttcagcttctgg, T7 primers: forward: gcgtaatacgactcactataggccacagggctgccactg, reverse: gcgtaatacgactcactataggaagtcgctggtgttaagcc. Primers for PRR5L were as follows: gene specific primers: forward: tcgtccattgtccagatgttg, reverse: agctgagggaagcacagttc, T7 primers: forward: gcgtaatacgactcactataggctcatcctgcagagtgttc, reverse: gcgtaatacgactcactataggagcctccgagccctcctg. For SIN1 knock down, a synthetic pool siRNA or the appropriate control pool siRNA (Dharmacon) were used as described [19].

Cell culture and transfections

HEK293 and HeLa cells were maintained in DMEM containing 10% fetal bovine serum. Small RNAi was transfected with INTERFERin (Polyplus transfection). For combined transfection of small RNAi and DNA, jetSI-ENDO (Polyplus transfection) was used. DNA was transfected using either lipofectamin (Invitrogen) or jetPEI (Polyplus transfection). All transfections were done according to the manufacturers' instructions, for 48 h for expression or small RNAi, or for 4 days in the case of pSuper-based siRNA. Cells were harvested with lysis buffer that contained 40mM HEPES pH 7.5, 120 mM NaCl, 1 mM EDTA, 0.3% CHAPS, supplemented with protease inhibitor cocktail (Roche). In the cases where phosphorylation was to be detected, the lysis buffer was also supplemented with10 mM NaF, 10 mM NaN3, 10 mM p-nitrophenylphosphate, 10 mM sodium pyrophosphate, and 10 mM beta-glycerophosphate. Lysates were incubated for 20 minutes on ice, then cleared by a spin at 600 g for 3 minutes. Supernatants were collected and used for immunoprecipitations, GST pull downs or immunoblots.

RT-PCR

RNA was purified using the RNAeasy mini kit (Qiagen), according to the manufacturer's instructions. One microgram of total RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen) and random nonamers (Sigma). The reverse transcription reaction was used as a template for PCR, with the PRR5L primers described above.

Immunoblotting

Protein extracts were prepared as described [14], resolved on SDS-PAGE and transferred to PVDF membranes (Immobilon-P, Millipore). Immunoblots were performed using the following antibodies: Rictor, Raptor (Bethyl); mTOR (Santa Cruz); S6K, phospho-S6K, Akt, phospho-Akt (Thr308), phospho-Akt (Ser473), PARP, cleaved PARP (Cell signaling); PRAS40 (Biosource; PRAS40 antibodies were previously described [37]); SIN1 (kind gift from Dr. Bing Su, University of Yale, CT). GST-PRR5L was detected with a GST antibody (GE Healthcare), since no antibody for PRR5L was available.

Apoptosis assay

RNAi experiments were performed by transfecting HeLa cells for 48 hours in a 96-well format. 48 hours after siRNA transfection, apoptosis was induced for the indicated times as described [56]. Apoptosis, in response to TNFα and cycloheximide treatment, was quantified by a cleaved PARP (cPARP) immunofluorescence assay (Cell Signaling). The assay was performed according to the manufacturer's instructions. Briefly, after apoptosis induction, cells were fixed with pre-chilled 100% methanol for 5min and then washed with 0.1% Triton X-100 and PBS sequentially. Cells were then incubated with anti-cPARP antibody (1/200 dilution) overnight at 4°C, washed and incubated for 1 hour with a mixed solution containing Alexa 568 goat anti-Rabbit antibody (1/500 dilution) and Hoechst (Invitrogen, 1/1000 dilution). Images were automatically taken by an ImageXpress Micro (Molecular Devices, Sunnyvale, USA). Apoptosis was quantified by automated image processing. The multi-wavelength cell scoring application module of the analysis software MetaXpress was used to quantify, at the single cell level, the intensity of cPARP staining (200 was used as the intensity above background for cPARP images). More than 6000 cells per condition were analyzed. For analysis by immunoblotting cPARP levels were detected by human or mouse specific antibodies (Cell Signaling). mTORC1 was inhibited by preincubation with 100 nM rapamycin for 1 h, and apoptosis was subsequently induced by TNFα/cycloheximide for 1.5 h in the presence of rapamycin.

Immunoprecipitation and GST pull down

Immunoprecipitations were performed as described [14]. Pull downs of GST-PRR5L were similar to immunoprecipitations, with glutathione-coupled beads (GE Healthcare).

Kinase assay

Kinase assays were performed as described [14].

SUPPORTING INFORMATION

Figure S1 Time course for apoptosis induction. HeLa cells were transfected with PRR5L or control siRNA and incubated for 48 h, followed by apoptosis induction for the indicated time spans by TNFalpha and cycloheximide. Cells were fixed and stained with cleaved PARP antibody, and the percent of apoptotic cells was quantified.

Found at: doi:10.1371/journal.pone.0001217.s001 (0.64 MB TIF)

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Author Contributions

Conceived and designed the experiments: MH KT PP. Performed the experiments: KT PP MK PJ. Analyzed the data: MH KT PP MK CA. Contributed reagents/materials/analysis tools: KM AC CA. Wrote the paper: MH KT PP.

REFERENCES

1. Wullschleger S, Loewith R, Hall MN (2006) TOR Signaling in Growth and Metabolism. Cell 124: 471–484.
2. Corradetti MN, Guan KL (2006) Upstream of the mammalian target of rapamycin: do all roads pass through mTOR? Oncogene 25: 6347–6360.
3. Hay N, Sonenberg N (2004) Upstream and downstream of mTOR. Genes Dev 18: 1926–1945.
4. Menand B, Desnos T, Nussaume L, Berger F, Bouchez D, et al. (2002) Expression and disruption of the Arabidopsis TOR (target of rapamycin) gene. Proc Natl Acad Sci U S A 99: 6422–6427.
5. Long X, Spycher C, Han ZS, Rose AM, Muller F, et al. (2002) TOR deficiency in C. elegans causes developmental arrest and intestinal atrophy by inhibition of mRNA translation. Curr Biol 12: 1448–1461.
6. Oldham S, Montagne J, Radimerski T, Thomas G, Hafen E (2000) Genetic and biochemical characterization of dTOR, the Drosophila homolog of the target of rapamycin. Genes Dev 14: 2689–2694.
7. Zhang H, Stallock JP, Ng JC, Reinhard C, Neufeld TP (2000) Regulation of cellular growth by the Drosophila target of rapamycin dTOR. Genes Dev 14: 2712–2724.
8. Brown EJ, Albers MW, Shin TB, Ichikawa K, Keith CT, et al. (1994) A mammalian protein targeted by G1-arresting rapamycin-receptor complex. Nature 369: 756–758.
9. Chiu MI, Katz H, Berlin V (1994) RAFT1, a mammalian homolog of yeast Tor, interacts with the FKBP12/rapamycin complex. Proc Natl Acad Sci U S A 91: 12574–12578.
10. Sabatini DM, Erdjument-Bromage H, Lui M, Tempst P, Snyder SH (1994) RAFT1: a mammalian protein that binds to FKBP12 in a rapamycin-dependent fashion and is homologous to yeast TORs. Cell 78: 35–43.
11. Kunz J, Henriquez R, Schneider U, Deuter-Reinhard M, Movva NR, et al. (1993) Target of rapamycin in yeast, TOR2, is an essential phosphatidylinositol kinase homolog required for G1 progression. Cell 73: 585–596.
12. Gangloff YG, Mueller M, Dann SG, Svoboda P, Sticker M, et al. (2004) Disruption of the mouse mTOR gene leads to early postimplantation lethality and prohibits embryonic stem cell development. Mol Cell Biol 24: 9508–9516.
13. Loewith R, Jacinto E, Wullschleger S, Lorberg A, Crespo JL, et al. (2002) Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. Mol Cell 10: 457–468.
14. Jacinto E, Loewith R, Schmidt A, Lin S, Ruegg MA, et al. (2004) Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. Nat Cell Biol 6: 1122–1128.
15. Sarbassov DD, Ali SM, Kim DH, Guertin DA, Latek RR, et al. (2004) Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. Curr Biol 14: 1296–1302.
16. Kim DH, Sarbassov DD, Ali SM, King JE, Latek RR, et al. (2002) mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. Cell 110: 163–175.
17. Hara K, Maruki Y, Long X, Yoshino K, Oshiro N, et al. (2002) Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. Cell 110: 177–189.
18. Frias MA, Thoreen CC, Jaffe JD, Schroder W, Sculley T, et al. (2006) mSin1 is necessary for Akt/PKB phosphorylation, and its isoforms define three distinct mTORC2s. Curr Biol 16: 1865–1870.
19. Yang Q, Inoki K, Ikenoue T, Guan KL (2006) Identification of Sin1 as an essential TORC2 component required for complex formation and kinase activity. Genes Dev 20: 2820–2832.
20. Jacinto E, Facchinetti V, Liu D, Soto N, Wei S, et al. (2006) SIN1/MIP1 maintains rictor-mTOR complex integrity and regulates Akt phosphorylation and substrate specificity. Cell 127: 125–137.
21. Shiota C, Woo JT, Lindner J, Shelton KD, Magnuson MA (2006) Multiallelic disruption of the rictor gene in mice reveals that mTOR complex 2 is essential for fetal growth and viability. Dev Cell 11: 583–589.
22. Guertin DA, Stevens DM, Thoreen CC, Burds AA, Kalaany NY, et al. (2006) Ablation in mice of the mTORC components raptor, rictor, or mLST8 reveals that mTORC2 is required for signaling to Akt-FOXO and PKCalpha, but not S6K1. Dev Cell 11: 859–871.
23. Kwiatkowski DJ (2003) Tuberous sclerosis: from tubers to mTOR. Ann Hum Genet 67: 87–96.
24. Crino PB, Nathanson KL, Henske EP (2006) The tuberous sclerosis complex. N Engl J Med 355: 1345–1356.
25. Zhang Y, Gao X, Saucedo LJ, Ru B, Edgar BA, et al. (2003) Rheb is a direct target of the tuberous sclerosis tumour suppressor proteins. Nat Cell Biol 5: 578–581.
26. Garami A, Zwartkruis FJ, Nobukuni T, Joaquin M, Roccio M, et al. (2003) Insulin activation of Rheb, a mediator of mTOR/S6K/4E-BP signaling, is inhibited by TSC1 and 2. Mol Cell 11: 1457–1466.
27. Avruch J, Hara K, Lin Y, Liu M, Long X, et al. (2006) Insulin and amino-acid regulation of mTOR signaling and kinase activity through the Rheb GTPase. Oncogene 25: 6361–6372.
28. Shaw RJ, Cantley LC (2006) Ras, PI(3)K and mTOR signalling controls tumour cell growth. Nature 441: 424–430.
29. Dennis PB, Jaeschke A, Saitoh M, Fowler B, Kozma SC, et al. (2001) Mammalian TOR: a homeostatic ATP sensor. Science 294: 1102–1105.
30. Inoki K, Zhu T, Guan KL (2003) TSC2 mediates cellular energy response to control cell growth and survival. Cell 115: 577–590.
31. Nobukuni T, Joaquin M, Roccio M, Dann SG, Kim SY, et al. (2005) Amino acids mediate mTOR/raptor signaling through activation of class 3 phosphatidylinositol 3OH-kinase. Proc Natl Acad Sci U S A 102: 14238–14243.
32. Yang Q, Inoki K, Kim E, Guan KL (2006) TSC1/TSC2 and Rheb have different effects on TORC1 and TORC2 activity. Proc Natl Acad Sci U S A 103: 6811–6816.
33. Burnett PE, Barrow RK, Cohen NA, Snyder SH, Sabatini DM (1998) RAFT1 phosphorylation of the translational regulators p70 S6 kinase and 4E-BP1. Proc Natl Acad Sci U S A 95: 1432–1437.
34. Brunn GJ, Fadden P, Haystead TA, Lawrence JC Jr (1997) The mammalian target of rapamycin phosphorylates sites having a (Ser/Thr)-Pro motif and is activated by antibodies to a region near its COOH terminus. J Biol Chem 272: 32547–32550.
35. Schmidt A, Bickle M, Beck T, Hall MN (1997) The yeast phosphatidylinositol kinase homolog TOR2 activates RHO1 and RHO2 via the exchange factor ROM2. Cell 88: 531–542.
36. Sarbassov DD, Guertin DA, Ali SM, Sabatini DM (2005) Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science 307: 1098–1101.
37. Kovacina KS, Park GY, Bae SS, Guzzetta AW, Schaefer E, et al. (2003) Identification of a proline-rich Akt substrate as a 14-3-3 binding partner. J Biol Chem 278: 10189–10194.
38. Sancak Y, Thoreen CC, Peterson TR, Lindquist RA, Kang SA, et al. (2007) PRAS40 is an insulin-regulated inhibitor of the mTORC1 protein kinase. Mol Cell 25: 903–915.
39. Vander Haar E, Lee SI, Bandhakavi S, Griffin TJ, Kim DH (2007) Insulin signalling to mTOR mediated by the Akt/PKB substrate PRAS40. Nat Cell Biol 9: 316–323.
40. Johnstone CN, Castellvi-Bel S, Chang LM, Sung RK, Bowser MJ, et al. (2005) PRR5 encodes a conserved proline-rich protein predominant in kidney: analysis of genomic organization, expression, and mutation status in breast and colorectal carcinomas. Genomics 85: 338–351.
41. Sarbassov DD, Ali SM, Sengupta S, Sheen JH, Hsu PP, et al. (2006) Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. Mol Cell 22: 159–168.
42. Edinger AL, Thompson CB (2004) An activated mTOR mutant supports growth factor-independent, nutrient-dependent cell survival. Oncogene 23: 5654–5663.
43. Tarek O, Wadewitz B, Hans U, Ortmann O (2006) Effects of a combined treatment with mTOR inhibitor RAD001 and tamoxifen in vitro on growth and apoptosis of human cancer cells. Gynecol Oncol 102: 292–299.
44. Teachey DT, Obzut DA, Cooperman J, Fang J, Carroll M, et al. (2006) The mTOR inhibitor CCI-779 induces apoptosis and inhibits growth in preclinical models of primary adult human ALL. Blood 107: 1149–1155.
45. Avellino R, Romano S, Parasole R, Bisogni R, Lamberti A, et al. (2005) Rapamycin stimulates apoptosis of childhood acute lymphoblastic leukemia cells. Blood 106: 1400–1406.
46. Hahn M, Li W, Yu C, Rahmani M, Dent P, et al. (2005) Rapamycin and UCN-01 synergistically induce apoptosis in human leukemia cells through a process that is regulated by the Raf-1/MEK/ERK, Akt, and JNK signal transduction pathways. Mol Cancer Ther 4: 457–470.
47. Beuvink I, Boulay A, Fumagalli S, Zilbermann F, Ruetz S, et al. (2005) The mTOR inhibitor RAD001 sensitizes tumor cells to DNA-damaged induced apoptosis through inhibition of p21 translation. Cell 120: 747–759.
48. Thimmaiah KN, Easton J, Huang S, Veverka KA, Germain GS, et al. (2003) Insulin-like growth factor I-mediated protection from rapamycin-induced apoptosis is independent of Ras-Erk1-Erk2 and phosphatidylinositol 3′-kinase-Akt signaling pathways. Cancer Res 63: 364–374.
49. Saito A, Narasimhan P, Hayashi T, Okuno S, Ferrand-Drake M, et al. (2004) Neuroprotective role of a proline-rich Akt substrate in apoptotic neuronal cell death after stroke: relationships with nerve growth factor. J Neurosci 24: 1584–1593.

50. Huang B, Porter G (2005) Expression of proline-rich Akt-substrate PRAS40 in cell survival pathway and carcinogenesis. Acta Pharmacol Sin 26: 1253–1258.
51. Shah OJ, Wang Z, Hunter T (2004) Inappropriate activation of the TSC/Rheb/mTOR/S6K cassette induces IRS1/2 depletion, insulin resistance, and cell survival deficiencies. Curr Biol 14: 1650–1656.
52. Ghosh S, Tergaonkar V, Rottlin CV, Correa RG, Bottero V, et al. (2006) Essential role of tuberous sclerosis genes TSC1 and TSC2 in NF-kappaB activation and cell survival. Cancer Cell 10: 215–226.
53. Clerenssen CA, Schroeder MJ, Shabanowitz J, Hunt DF, Pelo JW, et al. (2004) MAPKAP kinase 2 phosphorylates tristetraprolin on in vivo sites including Ser178, a site required for 14-3-3 binding. J Biol Chem 279: 10176–10184.
54. Gatlin CL, Eng JK, Cross ST, Detter JC, Yates JR III (2000) Automated identification of amino acid sequence variations in proteins by HPLC/microspray tandem mass spectrometry. Anal Chem 72: 757–763.
55. Liou J, Kim ML, Heo WD, Jones JT, Myers JW, et al. (2005) STIM is a Ca2+ sensor essential for Ca2+-store-depletion-triggered Ca2+ influx. Curr Biol 15: 1235–1241.
56. Kettritz R, Schneider J, Xu Y, Luft FC, Haller H (2002) TNF-alpha-accelerated apoptosis abrogates ANCA-mediated neutrophil respiratory burst by a caspase-dependent mechanism. Kidney Int 61: 502–515.

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Publications

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Kim ML, Jeong HG, Kasper CA, Arrieumerlou C. IKKα contributes to canonical NF-κB activation downstream of Nod1-mediated peptidoglycan recognition. *PLoS One*. 2010 Oct 15;5(10):e15371.

Park HC, Kim ML, Kim HS, Park JH, Jung MS, Shen M, Kang CH, Kim MC, Lee SY, Cho MJ, Chung WS, Yun DJ. Specificity of DNA sequences recognized by the zinc-finger homeodomain protein, GmZF-HD1 in soybean. *Phytochemistry*. 2010 Nov;71(16):1832-8.

Park HC, Kim ML, Kang YH, Jeong JC, Cheong MS, Choi W, Lee SY, Cho MJ, Kim MC, Chung WS, Yun DJ. Functional analysis of the stress-inducible soybean calmodulin isoform-4 (GmCaM-4) promoter in transgenic tobacco plants. *Mol Cells*. 2009;27(4):475-80.

Thedieck K, Polak P, Kim ML, Molle KD, Cohen A, Jenö P, Arrieumerlou C, Hall MN. PRAS40 and PRR5 Like Protein Are New mTOR Interactors that Regulate Apoptosis. *PLoS One*. 2007; 2(11):e1217

Galvez T, Teruel MN, Heo WD, Jones JT, Kim ML, Liou J, Myers JW, Meyer T. siRNA screen of the human signaling proteome identifies the PtdIns(3,4,5)P3-mTOR signaling pathway as a primary regulator of transferrin uptake. *Genome Biol*. 2007;8(7):R142.

Park HC*, Kim ML*, Lee SM, Bahk JD, Yun DJ, Lim CO, Hong JC, Lee SY, Cho MJ, Chung WS. Pathogen-induced binding of the soybean zinc finger homeodomain proteins GmZF-HD1 and GmZF-HD2 to two repeats of ATTA homeodomain binding site in the calmodulin isoform 4 (GmCaM4) promoter. *Nucleic Acids Res.* 2007;35(11):3612-23. (* These authors contributed equally to this work)

Heo WD, Inoue T, Park WS, Kim ML, Park BO, Wandless TJ, Meyer T. PI(3,4,5)P3 and PI(4,5)P2 lipids target proteins with polybasic clusters to the plasma membrane. *Science.* 2006; 314(5804):1458-61.

Liou J, Kim ML, Heo WD, Jones JT, Myers JW, Ferrell JE Jr, Meyer T. STIM is a Ca^{2+}-sensor protein essential for Ca^{2+}-store depletion triggered Ca^{2+}-influx: *Curr Biol.* 2005;15(13):1235-1241

Park HC, Kim ML, Kang YH, Jeon JM, Yoo JH, Kim MC, Park CY, Jeong JC, Moon BC, Lee JH, Yoon HW, Lee SH, Chung WS, Lim CO, Lee SY, Hong JC, Cho MJ. Pathogen- and NaCl-induced expression of the SCaM-4 promoter is mediated in part by a GT-1 box that interacts with a GT-1-like transcription factor. *Plant Physiol*. 2004;135(4):2150-61.

Kim CY, Koo YD, Jin JB, Moon BC, Kang CH, Kim ST, Park BO, Lee SY, Kim ML, Hwang I, Kang KY, Bahk JD, Lee SY, Cho MJ. Rice C2-domain proteins are induced and translocated to the plasma membrane in response to a fungal elicitor. *Biochemistry*. 2003;42(40):11625-33.