# Regulation of conformation and activity of nuclear NF-κB p65 by phosphorylation, chaperones and p65 DNA-binding

**Dissertation**

zur Erlangung des Doktorgrades
der Naturwissenschaften

-Dr. rer. nat.-

angefertigt am Institut für Biochemie
Fachbereich Medizin und dem Fachbereich Biologie und Chemie
Justus-Liebig-Universität Gießen

vorgelegt von

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Gießen, März 2014

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Maja Milanovic

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# Abbreviations

| | |
|---|---|
| % (v/v) | volume/volume percentage |
| % (w/v) | weight/volume percentage |
| ºC | degree Celsius |
| 3D | three-dimensional |
| 17-AAG | 17-N-allylamino-17-demethoxygeldanamycin |
| Ab | antibody |
| ABIN | A20 binding inhibitor of NF-κB 2 |
| ADP | adenosine diphosphate |
| AES ` | amino enhancer of split |
| ANK | ankyrin repeat |
| Apaf-1 | apoptotic protease activating factor 1 |
| Arg | arginine |
| ARF | alternate reading frame |
| Asp | aspartic acid |
| ATM | ataxia telangiectasia mutated |
| ATP | adenosine triphosphate |
| BAFFR | B-cell activating factor of the TNF family receptor |
| Bcl-xL | B-cell lymphoma-extra large |
| bp | base pairs |
| β-TrCP | β-transducin repeat-containing protein |
| $C_t$ | threshold cycle |
| CaMKIV | calmodulin-dependent protein kinase IV |
| CBP | CREB-(c-AMP-response element-binding protein) -binding protein |
| CC | coiled-coil |
| CCT | chaperonin-containing t-complex polypeptide 1 (TCP1) |
| CD | circular dichroism |
| cDNA | complementary DNA |
| CDK6 | cyclin dependent kinase 6 |
| ChIP | chromatin immunoprecipitation |
| CHIP | carboxyl-terminus of Hsp70 interacting protein |
| Chk1 | checkpoint kinase 1 |

| | |
|---|---|
| cIAP | cellular inhibitor of apoptosis protein |
| CIAP | calf intestine alkaline phosphatase |
| COMMD1 | copper metabolism MURR1 domain-containing protein 1 |
| CoREST | corepressor for REST (RE1 silencing TF) |
| Crm-1 | chromosomal region maintenance-1 |
| Cul2 | cullin-2 |
| DD | dimerisation domain |
| DISC | death-inducing signaling complex |
| DNA | deoxyribonucleic acid |
| ds | double-stranded |
| E3 | ubiquitin ligase enzyme |
| E2 | ubiquitin conjugating enzyme |
| Elk-1 | ETS domain-containing protein1 |
| ELKS | protein rich in glutamate, leucine, lysine and serine |
| EMSA | electrophoretic mobility shift assay |
| EPR | electron paramagnetic resonance |
| FADD | Fas-associated protein with death domain |
| FBXL11 | F-box and leucine-rich repeat protein 11 |
| FoxO1 | forkhead box O1 |
| GA | geldanamycin |
| GCN5 | general control nonderepressible 5 |
| GFP | green fluorescent protein |
| GLP | G9A-like protein |
| Glu | glutamic acid |
| GR | glucocorticoid receptor |
| GRR | glycine-rich region |
| GSK-3β | glycogen-synthase kinase-3beta |
| h | hour |
| HA | hemagglutinin |
| HLH | helix-loop-helix |
| HDAC | histone deacetylase |
| HEK | human embryonic kidney |
| His | histidine |
| HOIL-1 | heme-oxidized IRP2 ubiquitin ligase-1 |

| | |
|---|---|
| HOIP | HOIL-1 interacting protein |
| HOP | Hsp organizing protein |
| HSF-1 | heat shock transcription factor-1 |
| Hsp | heat shock protein |
| IAP | inhibitor of apoptosis |
| IB | immunoblotting |
| Ig | immunoglobulin |
| ID | intrinsic disorder |
| IFNβ | interferon β |
| IL-1 | interleukin-1 |
| IL-1R | interleukin-1 receptor |
| IκB | inhibitor of NF-κB |
| IKK | IκB kinase |
| IP | immunoprecipitation |
| IRAK1 | IL-1 receptor associated kinase 1 |
| IRF3 | interferon regulatory transcription factor |
| ITC | Isothermal calorimetry |
| kb | kilo base |
| KD | kinase domain |
| kDa | kilo dalton |
| Leu | leucine |
| LPS | lipopolysaccharide |
| LTβR | lymphotoxin-β receptor |
| LUBAC | linear ubiquitin chain assembly complex |
| LZ | leucine zipper |
| mAb | monoclonal antibody |
| Mal | MyD88 adaptor like |
| MDM2 | mouse double minute 2 homolog |
| min | minutes |
| MEF | mouse embryonic fibroblast |
| MEKK | mitogen-activated protein kinase kinase kinase |
| MS | mass spectrometry |
| MSK-1 | mitogen- and stress-activated protein kinase-1 |
| MYBBP1a | Myb-binding protein 1a |

| | |
|---|---|
| MyD88 | myeloid differentiation factor 88 |
| NBD | NEMO-binding domain |
| NCoR | nuclear receptor corepressor |
| NEF | nucleotide exchange factor |
| NEMO | NF-κB essential modulator |
| NES | nuclear export signal |
| NF-κB | nuclear factor-κB |
| NFAT | nuclear factor of activated T cells |
| NIK | NF-κB-inducing kinase |
| Ni-NTA | nickel-nitrilotriacetic acid |
| NLS | nuclear localization signal |
| NMR | nuclear magnetic resonance |
| NSD1 | nuclear receptor-binding SET domain protein 1 |
| NTD | N-terminal domain |
| Nurr1 | nuclear receptorrelated-1 protein |
| Pro | proline |
| pAb | polyclonal antibody |
| P-TEFb | positive transcription elongation factor b |
| PARP-1 | poly (ADP-ribose) polymerase-1 |
| PAMP | pathogen-associated molecular pattern |
| PCR | polymerase chain reaction |
| PDLIM2 | PDZ and LIM domain 2 |
| PEST | proline-glutamic acid-serine-threonine sequence |
| PHF20 | PHD finger protein 20 |
| PIASy | protein inhibitor of activated STAT4 |
| Pim-1 | proviral integration site for the Moloney-murine leukemia virus-1 |
| Pin-1 | peptidyl-prolyl cis-trans isomerase NIMA-interacting-1 |
| PKAc | catalytic subunit of protein kinase A |
| PKC | protein kinase C |
| PKCζ | protein kinase C, zeta |
| PMA | phorbol myristoylacetate |
| PML | promyelocytic leukemia protein |
| polyUb | polyubiquitination |
| PP2A | protein phosphatase 2A |

| | |
|---|---|
| PPA4 | protein phosphatase 4 |
| PRMT5 | protein arginine methyltransferase 5 |
| PTM | posttranslational modification |
| qRT-PCR | quantitative real-time PCR |
| RANK | receptor activator of NF-κB |
| RDH | Rel homology domain |
| RIP1 | receptor interacting protein 1 |
| RNA | ribonucleic acid |
| RNAi | RNA interference |
| RNA Pol II | RNA polymerase II |
| RSK-1 | ribosomal protein S6 kinase 1 |
| SAP | Shrimp alkaline phosphatase |
| sec | seconds |
| SETD6 | SET domain-containing protein 6 |
| Ser | serine |
| SET | suppressor of variegation-enhancer of zeste-trithorax |
| Seq | sequencing |
| shRNA | small-hairpin RNA |
| SHARPIN | SHANK-associated RH domain interacting protein |
| SMRT | silencing mediator for retinoic acid receptor and thyroid hormone receptor |
| SOCS-1 | suppressor of cytokine signaling-1 |
| SPINE-D | sequence based prediction with integrated neural network for disordered residues |
| SPR | surface plasmon resonance |
| SRC | steroid receptor cofactors |
| STAT | signal transducer and activator of transcription |
| TAB | TAK1 binding protein |
| TAD | transactivation domain |
| TAFII31 | TATA-binding-protein-associated factor II31 |
| TAK1 | transforming growth factor beta-activated kinase 1 |
| TANK | TRAF family member-associated NF-κB activator |
| TAZ1 | transcriptional adaptor zinc binding 1 |
| TBK1 | TANK-binding kinase 1 |
| TF | transcription factor |

| | |
|---|---|
| Thr | threonine |
| Tip60 | 60 kDa trans-acting regulatory protein of HIV type 1-interacting protein |
| TIR | Toll/interleukin-1 receptor |
| TIRAP | TIR-domain-containing adaptor protein |
| TLR | Toll-like receptors |
| TNF | tumor necrosis factor |
| TNFR | TNF receptor |
| TRADD | TNF receptor associated protein with a death domain |
| TRAF | TNF receptor associated factor |
| TRAM | TRIF-related adapter molecule |
| TRIF | TIR domain-containing adapter-inducing IFN-β |
| TRP | tetratricopeptide repeat |
| TSS | transcription start site |
| Tyr | tyrosine |
| Ub | ubiquitin |
| UBAN | ubiquitin binding in ABIN and NEMO |
| Ubc13 | ubiquitin conjugating enzyme 13 |
| Uev1A | E2 variant 1 isoform A |
| ULD | ubiquitin-like domain |
| uPA | urinary plasminogen activator |
| UV | ultraviolet light |
| VER | VER155008 |
| WIP1 | wild-type p53-induced phosphatase |
| YFP | yellow fluorescent protein |
| ZF | zinc finger |

# 1. Introduction

## 1.1 The NF-κB transcription factor

NF-κB (nuclear factor-κB) is a collective term for a family of eukaryotic transcription factors (TFs) that play a critical role in inflammation, immunity, cell proliferation, differentiation and survival. It exists in all cell types with a nucleus [1, 2]. NF-κB is composed of various combinations of different DNA-binding subunits: p105/p50 (NF-κB1), p100/p52 (NF-κB2), RelA (p65), RelB and c-Rel [2-4] (Fig.1.1). All of subunits possess a homologous sequence in their N-termini referred to as the Rel homology domain (RHD). This region is approximately 300 amino acids long and is responsible for critical functions including dimerisation, DNA binding, nuclear localization and association with a family of inhibitory proteins called inhibitors of κB (IκBs). The RHD can be divided into three structural regions: the N-terminal domain (NTD) involved in binding to DNA, the dimerisation domain (DD) and the nuclear localization signal (NLS). Between the NTD and the DD there is a short flexible region which participates in NF-κB DNA binding. The DD alone mediates the association of individual NF-κB subunits to form combinatorial dimers. The Rel/NF-κB family members can be grouped into two classes (Fig. 1.1, upper panel). The first class consists of p105 and p100 precursor proteins which are proteolytically processed to the NF-κB subunits p50 and p52, respectively. The mature p50 and p52 proteins contain the RHD followed by a 23-amino acid glycine-rich region (GRR), a region that is essential for directing the cleavage and proteolytic processing of a long IκB-like C-terminal part of the precursors [5]. The other class consists of p65, RelB and c-Rel which all contain transactivation domains (TADs) in their C-termini. The TAD regions are not conserved between the NF-κB subunits [6, 7]. They are rather functionally defined, as they activate transcription by recruitment of transcriptional coregulators and components of the basal transcriptional machinery [4]. Due to the lack of C-terminal TADs, NF-κB dimers composed only of p50 and/or p52 subunits are transcriptionally inactive [8-10].

### 1.1.1 The Rel homology domain

To date, X-ray crystal structures of the DNA-bound RHDs of the p50/p50, p52/p52 and p65/65 homodimers are known [10-13]. The structure of the p50/p65 heterodimer-DNA complex has also been reported [14].

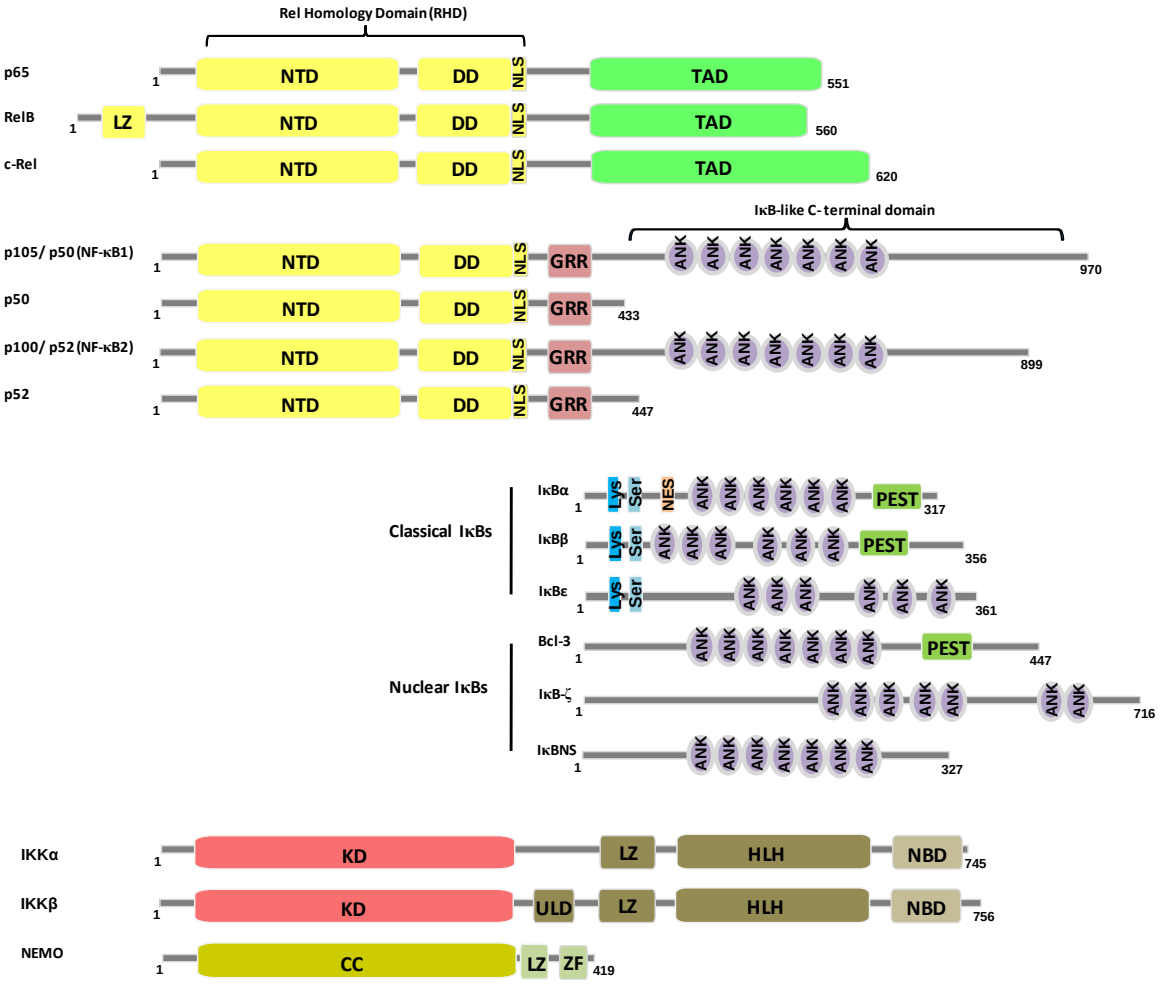



**Figure 1.1 Schematic representations of NF-κB, IκB and IκB kinase (IKK) family proteins.** The upper panel represents subunits of the NF-κB TF family. In the middle panel are members of the IκB protein family. The lower panel represents subunits of the IKK complex. Abbreviations: LZ (leucine zipper), ANK (ankyrin repeat), NES (nuclear export signal), PEST (proline-glutamic acid-serine-threonine sequence), Lys (lysine), Ser (serine), KD (kinase domain), ULD (ubiquitin (Ub)-like domain), HLH (helix-loop-helix), NBD (NEMO (NF-κB essential modulator)-binding domain), CC (coiled-coil), ZF (zinc finger).

The crystal structure of the murine p65 RHD (construct containing residues 9-291) bound to a specific DNA target revealed that the RHD region is folded into two immunoglobulin (Ig)-like domains which are connected by a 10 amino acid long flexible region [13]. The N-terminal Ig-like domain is responsible for sequence-specific DNA recognition. The C-terminal Ig-like fold is responsible for subunit dimerisation and non-specific DNA binding.

Unlike most DNA-binding proteins, which use α-helices for base-pair recognition, Rel family dimers use loops from the edge of the N- and C- terminal parts of the RHD to mediate DNA contacts [14]. In case of p65 subunit, five loops are involved in DNA binding (Fig. 1.2). p65 makes DNA base-specific contacts via Tyr36, Glu39, Arg33 and Arg35 that arise from the loop L1 (residues 30-50) and Arg187 located in the loop L3 which links the N-terminal domain to the DD. The loop L2 is located in the N-terminal domain, while loops L4 and L5 arise from the DD. Amino acids from the L2, L4 and L5 loops contact only the phosphodiester backbone of the target DNA [15].

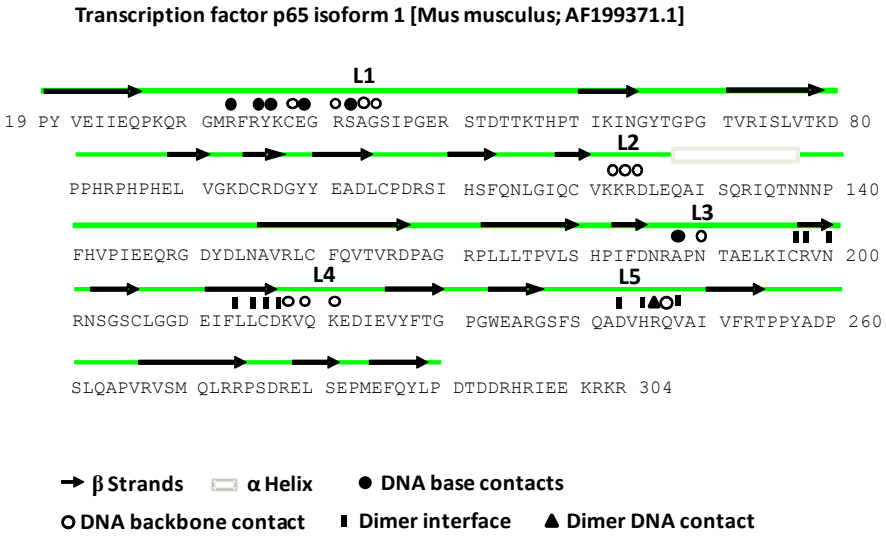



**Figure 1.2 Sequence of a murine p65 RHD with shown secondary structure.** The scheme is modified from [15].

***The DNA target sequence*** - NF-κB recognizes 9-11 bp (base pairs) long double-stranded (ds) DNA-elements termed as κB elements. They are often located within promoters and enhancers of NF-κB target genes[16, 17], but chromatin immunoprecipitation (ChIP) experiments with parallel DNA sequencing (ChIP-Seq) identified κB sites in exons and intergenic space as well [18-22]. The first NF-κB binding site was identified as a B-cell specific element in the intronic enhancer of the Ig κ light chain gene, with the sequence 5'-GGGACTTTCC-3' [23]. Comparison of several different κB sequences recognized by the NF-κB dimers, allowed the delineation of a consensus sequence 5'-GGGRNWYYCC-3', where R denotes a purine base, N means any base, W stands for adenine or thymine and Y represents a pyrimidine base [24]. Hundreds of such sequences have been confirmed experimentally. Non-consensus κB sites that hold a significant variation in comparison to

the original consensus κB sequence, were also identified [18-22]. In general, the κB site is pseudo-symmetric and each NF-κB monomer binds to one DNA half site. The NF-κB p50 and p52 subunits prefer the first half of the κB sequence that begin with 5'-GGG and is five bp in length. The second half of the κB DNA is four bp in length (5'-YYCC-3') and is preferentially occupied by the TAD-containing subunits (p65, RelB or c-Rel). Homodimers of p50 or p52 would bind optimally to an 11 bp κB DNA, while p65, RelB and c-Rel prefer binding to 9 bp κB DNA [4, 13].

***The NF-κB DD*** -The DD domain of NF-κB consists of approximately 100 amino acids near the C-terminal end of the RHD. The corresponding segment of p65 includes 101 residues from Thr191 to Asp291 [25]. About 12-14 residues of each monomer are directly involved in formation of a dimer interface. A close inspection of these structures revealed that differential selectivity and stability of NF-κB dimers are influenced by variations in residues across the dimer interface and by variations of residues located outside of the dimer interface that influence folding stability of the DD [4].

***The NLS*** **–** The NF-κB NLS is located next to the DD and is recognized by the stretch of the basic amino acids KRKR ($^{301}$KRKR$^{304}$, respectively for human p65). It regulates the nuclear localization of the NF-κB dimers via recognition by the import receptor [26]. The p65 NLS peptide and surrounding sequence (residues 293-321) are unfolded in the free state, while they gain structure upon binding to IκBα [27].

### 1.1.2 The p65 TAD

The three NF-κB subunits p65, RelB and c-Rel contain non-homologous TADs in their C-termini which enables them to trigger gene expression [7]. As the p65 subunit is the most abundantly studied member of this protein family, the architecture and properties of p65 C-terminal TAD will be described in more detail. The C-terminal 120 amino acids contain two strong and fully independent TADs: TAD1 in the last C-terminal 30 amino acids (residues 521-551 of human p65) and TAD2 directly adjacent to TAD1 (residues 428-521 of human p65) (Fig. 1.3) [28-31]. NMR (nuclear magnetic resonance) studies of the polypeptide corresponding to p65 amino acids 428-551, which comprises both TAD1 and TAD2, showed that the p65 transactivating C-terminus is unstructured under physiological conditions [28]. TAD1 and TAD2 of p65 belong to the class of acidic TAD domains. They are characterized by the presence of evolutionary conserved regions (TAD1 and TAD1'

located within TAD2, Fig. 1.3), which contain high percentages of acidic and hydrophobic amino acid residues and can form amphipatic α-helical structures in the hydrophobic solvent [6, 32]. The N-terminus of TAD2 possesses a mini-LZ (Leu436, Leu443 and Leu450) which contributes to the transactivation activity of the TAD2 subdomain [28]. The N-terminus of TAD2 also harbors a NES-like sequence [33].

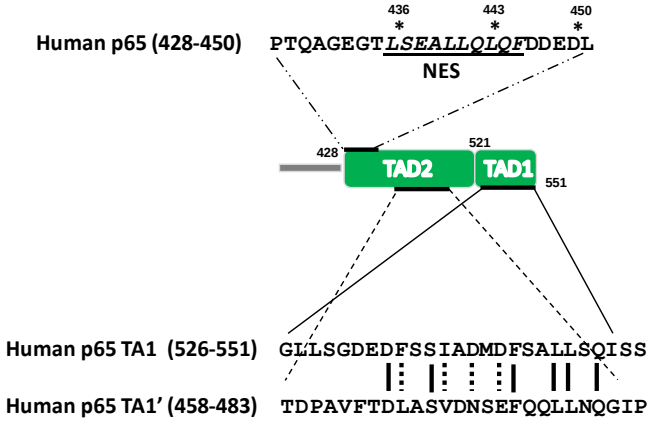



**Figure 1.3 Schematic representation of human p65 C-terminal TAD.** Upper: underlined NES-like sequence in p65, stars indicate lysines of the mini LZ-like motif. Lower: the TAD2region with homology to TAD1 is designated as TAD1'. Identical positions are shown by *solid bars*, the conserved hydrophobic amino acids by *dashed bars*. Figure modified from [6].

## 1.2. IκBs and IKKs as components of the NF-κB signaling pathways

### 1.2.1. The IκB protein family

The activity of NF-κB dimers is directly controlled by a set of IκBs through the formation of stable IκB-NF-κB complexes. Within those complexes, IκB protein masks the NLS of NF-κB subunits, thereby preventing NF-κB translocation into the nucleus. IκBs are the coevolved functional partners of NF-κB and regulate NF-κB-dependent gene expression under a variety of different conditions [34]. The IκB protein family is characterized by the presence of six to seven conserved ANK motifs which mediate IκB interaction with the RHD of NF-κB dimers [4]. These motifs are known to play an important role in protein-protein interactions while lacking any enzymatic activity [35]. The IκB protein family contains the classical IκBs (IκBα, IκBβ and IκBε), the non-classical IκBs (NF-κB precursors p105 and p100) and the nuclear IκBs ( Bcl-3, IκBζ, IκBNS and IκBη) (Fig. 1.1) [36]. Classical IκBs are defined by the presence of six ANKs [36]. The N-terminal

sequences of typical IκBs display an unfolded structure and contain the signal response domain or degron with the conserved serine residues that undergo stimulus-induced phosphorylation and subsequent rapid ubiquitination-mediated proteasomal degradation. Typical IκBs and p105 and p100 precursor proteins contain a 10 amino acid long acidic region also referred to as the PEST sequence that is involved in basal protein turnover [4, 36, 37]. The complete set of ANKs and the PEST sequence are necessary and sufficient for high affinity binding of IκBα, Bcl-3 or p105 to the dimerised RHD [38, 39].

#### 1.2.1.1 IκB-dependent regulation of NF-κB activity

IκBα is a 37 kDa protein and the best characterized member of the IκB family. IκBα regulates rapid and transient induction of NF-κB activity. The crystal structure of IκBα bound to the p65/p50 heterodimer revealed that one IκBα molecule binds to a NF-κB dimer and masks only the NLS of p65, whereas the NLS of p50 remains exposed [40, 41]. The exposed NLS from NF-κB subunits together with the NES of IκBα leads to constant shuttling of IκBα–NF-κB complexes between the nucleus and cytosol [42-44]. Degradation of IκBα eliminates the contribution of the NES in IκBα and leads to exposure of the previously masked NLS of p65, thus allowing accumulation of free DNA-binding NF-κB dimer in the nucleus [45] and its binding to κB elements. Due to the presence of κB sites in the *IκBα* promoter, the activation of NF-κB causes the upregulation of IκBα mRNA. Newly synthesized IκBα dissociates NF-κB from the DNA and promotes transcriptional termination [46-48]. The mechanism of nuclear translocation of *de novo* synthesized IκBα is not fully understood. One possibility is that the second ANK of IκBα serves as a non-classical NLS [49], while other studies suggested a "piggy-back" mechanism by which IκBα binds to an unidentified associated protein and utilizes its NLS [50, 51]. IκBα shuts off NF-κB-dependent transcription by two different mechanisms: via binding to the temporarily free nuclear NF-κBs or through direct interaction with NF-κB-DNA complexes [52-54]. Real-time binding kinetics experiments revealed that IκBα increases the dissociation rate of NF-κB from DNA with the help of the weakly folded fifth and sixth ANKs as well as the PEST sequence and that the interaction-dependent folding of the C-terminal ANKs is essential to optimally compete with DNA [53]. The initial binding of the fully structured N-terminal ANKs of IκBα to the NLS of the p65 subunit is followed by the association of the weakly folded C-terminal ANKs to dimerisation domains of p65 and p50. This leads to the complete folding of the C-terminal ANKs,

allowing the correct positioning of the negatively charged PEST sequence to efficiently displace IκBα from DNA [54]. The nuclear export of IκBα-NF-κB complex requires the IκBα NES between residues 45-54. This mechanism was suggested to be required for proper termination of TNF (tumor necrosis factor)-induced NF-κB activity [42].

IκBβ is a 45 kDa protein that is constitutively phosphorylated in resting cells [55]. In contrast to IκBα, IκBβ lacks the NES and is not an NF-κB target gene, even though the IκBβ promoter contains a κB site [56, 57]. Nevertheless, IκBβ can be resynthesized following stimulation with LPS (lipopolysaccharide) for four hours in a stimulus-dependent manner and appears as a hypophosphorylated protein which enters the nucleus and forms a stable complex with DNA-bound NF-κB [58, 59]. Therefore, besides its role as a cytoplasmic NF-κB inhibitor in resting cells, IκBβ also acts as a target gene-specific nuclear coactivator in TLR (Toll-like receptor) signaling.

The specific role of the 45 kDa protein IκBε is less well established. It is most likely that the combined action of IκBα and IκBε is required to regulate distinct expression dynamics of NF-κB-dependent target genes [34]. Stimulus-induced degron phosphorylation and subsequent ubiquitination-mediated proteasomal degradation of IκBε occurs with slower kinetics in comparison to IκBα and IκBβ. The transcription of the *IκBε* gene is NF-κB dependent but the resynthesis of IκBε occurs with a significantly delayed kinetics compared to IκBα [60]. Also, the nuclear import of IκBε occurs with a lower efficiency compared to IκBα. The nuclear export is mediated by a short NES-like sequence located between residues 343-352 within IκBε [61].

The C-termini of p100 and p105contain a long IκB-like domain containing seven ANKs, which blocks nuclear translocation and DNA-binding of the NF-κB precursor proteins [62-64]. The p105 precursor undergoes IKKβ-mediated phosphorylation at Ser927 and Ser932and subsequent ubiquitination-dependent proteolytic degradation, releasing the NF-κB p50-containing dimers [65]. The precursor p100 is phosphorylated by IKKα at conserved Ser866 and Ser870 and this promotes p100 polyubiquitination (polyUb) and processing by the proteasome, generating active p52 [66, 67]. The NF-κB precursor proteins are responsible for the inhibition of nearly half of the NF-κB dimers in resting cells. The precursors can assemble more than one NF-κB dimer into a high-molecular-weight complex which might function as a dynamic buffering system for NF-κB subunits

that are not bound to typical IκBs [68]. As targets of NF-κB, the precursor proteins p105 and p100 together with a newly synthesized classical IκBs serve to block NF-κB activity [69].

The atypical IκBs Bcl-3, IκBζ, IκBNS and IκBη are located in the nucleus and exhibit a variety of functions. In resting cells they display low expression levels, but upon stimulation with NF-κB-inducing agents their expression increases significantly [70-72]. They prevent the degradation of DNA-bound NF-κB dimers by competing with cytoplasmic IκBs for binding and provide interactions with other TFs. They can transactivate or inhibit transcription and provide a fine-tuning mechanism for late NF-κB-dependent gene regulation [73]. For example, Bcl-3 exhibits a coactivator function via interaction with DNA-bound p50 homodimer [74, 75] and provides an interaction platform for diverse multi-protein complexes involved in transcriptional regulation of NF-κB target genes [76-78].

### 1.2.2. The IKK complex

The common feature of all signaling pathways leading to NF-κB activation is the induction of IKKs. The IKK complex consists of two highly homologous kinases IKKα/IKK1 and IKKβ/IKK2 as well as the regulatory subunit IKKγ/NEMO [79] (Fig.1.1). IKKα and IKKβ are serine/threonine kinases characterized by the presence of an N-terminal kinase domain, followed by an ULD, a LZ and the C-terminal HLH domain. The C-terminus contains a stretch of six amino acids termed the NBD, which mediates interaction of IKKα/β with NEMO. In addition, IKKα contains a putative NLS [80] which possibly allows its translocation to the nucleus. NEMO is not related to IKKα and IKKβ and contains a CC, LZ and a C-terminal ZF-like domain [36] (Fig. 1.1). The activation of IKK proteins is mediated by phosphorylation of either IKKα or IKKβ at two specific serine residues (Ser177 and Ser181 for IKKα, and Ser176 and Ser180 for IKKβ) within the activation loop of the catalytic domain, yet its regulation is poorly understood [79]. Tang *et al.* showed that the activation of IKKβ depends on ligand-induced homotypic interactions between IKKβ molecules that result in its phosphorylation and consequently IKK activation [81]. On the other hand, it has been shown that TAK1 (transforming growth factor beta-activated kinase 1) directly phosphorylates IKKβ within the activation loop, leading to activation of the IKK complex [82]. NEMO acts as a scaffold protein that promotes the

assembly of the IKK complex and contributes to the recruitment of the IKK complex to upstream signaling molecules [84-86]. IKK proteins, although similar in structure, have relatively distinct substrates and functions that relate to the existence of different NF-κB activation pathways.

## 1.3 The NF-κB activating pathways

The NF-κB activating pathways can be grouped depending on the set of stimuli and signal transducing molecules as:

- The canonical NF-κB activation pathway
- The non-canonical NF-κB activation pathway
- The alternative NF-κB activation pathway

### 1.3.1 The canonical NF-κB pathway

Activation of the canonical pathway occurs in response to inflammatory cytokines, such as TNF and interleukin-1 (IL-1). These cytokines are recognized by corresponding membrane receptors: the TNF receptor (TNFR) and interleukin-1 receptor (IL-1R) [87]. The canonical pathway is also activated in response to PAMPs (pathogen-associated molecular patterns) such as LPS, flagellin, viral dsRNA and unmethylated CpG motifs recognized by TLRs. NF-κB activated by canonical pathway is involved in the control of innate immune responses and apoptosis [88, 89]. Depending on the nature of the stimulus and activated receptor, posttranslational modifications (PTMs) of signaling cascade components lead to the activation of the IKK complex. These modifications include ubiquitination of NEMO and phosphorylation of two serine residues in the activation loop of IKKβ [85, 90]. In most of the stimulus-initiated canonical pathways, IKKβ is necessary and sufficient for phosphorylation of IκBα at residues Ser32 and Ser36 and IκBβ at Ser19 and Ser23 [91]. This site-specific phosphorylation leads to IκBα/β ubiquitination, which tags IκBs for degradation by the proteasome [92]. Free DNA-binding p50/p65 dimers rapidly translocate to the nucleus within several minutes (min) [69]. Full activation of gene expression requires a number of further PTMs of the p65 subunit, including phosphorylation, acetylation, ubiquitination, methylation and prolyl-isomerisation [93, 94].

#### 1.3.1.1 The TNF-induced canonical NF-κB pathway

The cytokine TNF exerts its function by binding as a trimer to TNF receptor 1 (TNFR1), a member of the TNF receptor superfamily. Upon ligand binding, TNF receptors trimerize and the TNFR1 death domains associate to function as a docking site for the adaptor protein TRADD (TNF receptor associated protein with a death domain), the E3 (Ub ligase enzyme) ligase TRAF2 (TNF receptor associated factor-2), TRAF5 and the kinase RIP1 (receptor interacting protein 1). Anti-apoptotic regulators E3 ligases cIAP1 (cellular inhibitor of apoptosis protein 1) and cIAP2 are recruited to the TNFR complex via TRAF2/5 [95, 96]. TNFR1-associated RIP1 can be modified by various type of Ub chain modifications: Lys63-linked [97-99], Lys48-linked [100], Lys11-linked[101] and linear polyUb chains [102]. cIAP-mediated Lys63-polyUb chains recruit the IKK complex via binding of the Ub-binding domain of NEMO. Also, NEMO selectively binds linear Ub chains via its UBAN (Ub binding in ABIN (A20 binding inhibitor of NF-κB 2) and NEMO) motif, which was found important for the NF-κB activation by TNF [103]. RIP1 linear ubiquitination induces the recruitment of the LUBAC (linear ubiquitin chain assembly complex), a Ub-ligase complex composed of proteins HOIL-1 (heme-oxidized IRP2 ubiquitin ligase 1), HOIP (HOIL-1 interacting protein) and SHARPIN (SHANK-associated RH domain interacting protein) [102, 104]. LUBAC conjugates linear polyUb chains to NEMO and thereby regulates the NF-κB pathway since in the absence of LUBAC components the NF-κB signalling was attenuated [105, 106]. RIP1-attached Lys63-polyUb chains serve as a binding platform for TAK-TAB2/3 (TAK1 binding proteins 2 and 3) complex [107]. TAB2/3 and TAB1 are regulatory proteins for TAK1 which are constitutively associated with this kinase. TAB2/3 binding to the RIP1-attached Lys63-polyUb chain brings TAK1 and the IKK complex in close proximity which is required for the TAK1-mediated phosphorylation of IKKβ and activation of the IKK complex [91, 108-110] (Fig. 1.4).

TNFR1 signaling also involves the formation of a signaling complex which leads to the activation of apoptosis. At later time points after TNF stimulation, TRADD, RIP1 and TRAF2 dissociate from TNFR1. The liberated death domain from TRADD binds to the death domain-containing adaptor protein FADD (Fas-associated protein with death domain). FADD recruits Caspase-8 and forms a cytoplasmic complex DISC (death-inducing signaling complex) which leads to the cleavage and activation of Caspase 8 and to the induction of apoptosis [111, 112] (Fig. 1.4).

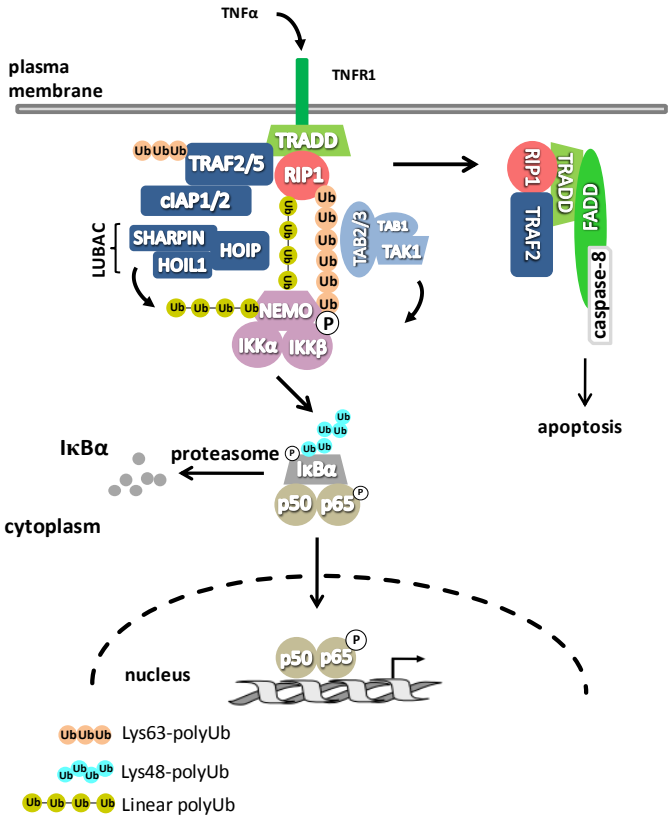



**Figure 1.4 The canonical TNFR1-mediated signaling pathway to NF-κB.** TNF-induced NF-κB signaling pathway mediated by the regulatory ubiquitination of RIP1 and activation of the IKK complex via LUBAC and TAK1/TABs complexes that are attached to RIP1 polyUb chains. TNF also induces the formation of apoptosis signaling complexes. Scheme is modified from [113].

### 1.3.1.2 The IL-1 and LPS-induced canonical NF-κB pathway

NF-κB activation in response to cytokine IL-1 or PAMPs is mediated via IL-1R or TLRsrespectively (Fig. 1.5). Upon binding of IL-1 to the IL-1R1, the adaptor protein MyD88 (myeloid differentiation factor 88) is recruited to the receptor via its own TIR (Toll/interleukin-1 receptor) domain [114, 115]. This leads to the assembly of a signaling complex that includes the serine/threonine kinase IRAK1 (IL-1 receptor associated kinase-1), IRAK4 and the E3 ligase TRAF6 (TNF receptor associated factor-6) [116, 117]. IRAK4 becomes activated by intramolecular auto-phosphorylation within its activation loop [118] and phosphorylates IRAK1. This further promotes dissociation of IRAK1 and TRAF6 from MyD88 [119, 120]. The E3 ligase β-TrCP (β-transducin repeat-containing protein) mediates Lys48-linked IRAK1 polyubiquitination (Lys48-polyUb) and subsequent IRAK1 degradation which has a critical role in dissociation of TRAF6 from IRAK1 [121]. In the cytoplasm, TRAF6 interacts with Ubc13 (Ub conjugating enzyme 13) and Uev1A

(E2 variant 1 isoform A), resulting in the regulatory ubiquitination of TRAF6 [122]. TRAF6-attached Lys63-polyUb chains provide a platform for recruitment of the TAK1-TABs, the LUBAC and IKK complex via NEMO. Finally, TAK1 phosphorylates IKKβ, leading to the activation of IKK complex and NF-κB [123, 124].

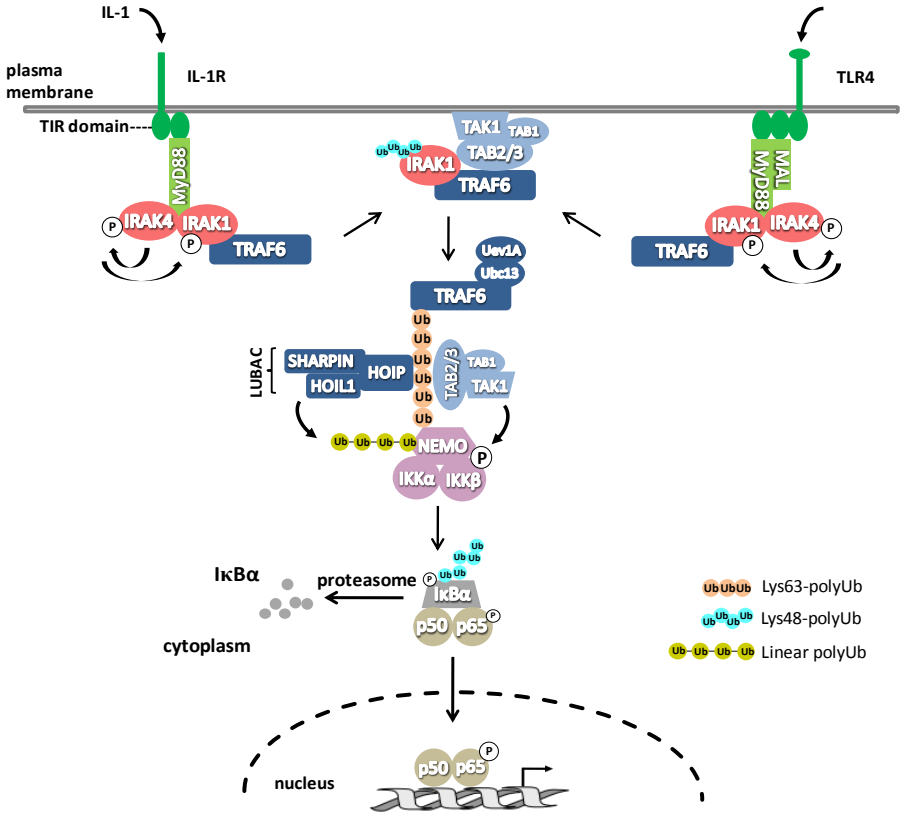



**Figure 1.5 Canonical IL-1R- and TLR4-mediated signaling pathways to NF-κB.** IL-1or LPS-induced NF-κB signaling mediated by the TRAF6 regulatory ubiquitination and subsequent IKK complex activation via TAK1/TABs and LUBAC. Scheme modified from [113, 125, 126].

Upon LPS stimulation, the signaling pathway originates from TLR4. In case of TLR4 signal transmission, MyD88 is recruited by interaction with TIRAP (TIR-domain-containing adaptor protein), also known as MyD88 adaptor like (Mal) [127, 128] and the following steps are the same as described for the IL-1-induced canonical NF-κB pathway (Fig.1.5). TLR4-mediated NF-κB activation can also be MyD88-independent. In such a case it involves the recruitment of TRAM (TRIF-related adapter molecule) and TRIF (TIR domain-containing adapter-inducing IFN (interferon)-β) [129]. TRIF associates with TRAF6 and RIP1 and activates the IKK complex via the Ub-activated TAK1/TAB complex [130].

### 1.3.2 The non-canonical and alternative NF-κB signaling pathways

The non-canonical NF-κB pathway is mediated by a specific subset of TNFR superfamily members including: LTβR (lymphotoxin-β receptor), BAFFR (B-cell activating factor of the TNF family receptor), CD40 and CD27 receptors, RANK (receptor activator of NF-κB) receptor or TNFR2. The key feature of the non-canonical pathway is the processing of the precursor p100 protein by the proteasome. This processing results in the release of the NF-κB subunit p52 and generation of p52-containing NF-κB dimers, in most cases p52/RelB heterodimers [131]. In comparison to the canonical pathway, this pathway is independent of IKKβ or NEMO, whereas the activation of NIK (NF-κB-inducing kinase) and IKKα are essential. For example, in response to CD40 ligand, NIK is activated and phosphorylates IKKα. Activated IKKα phosphorylates p100 at Ser866 and Ser870, leading to processing of precursor p100 to mature p52 and nuclear translocation of p52/RelB dimer [132-135] (Fig. 1.6A).

Activation of the NF-κB response can be induced by alternative mechanisms which are characterized by IKK activity in a manner distinct from those found in canonical and non-canonical pathways. Alternative pathways of NF-κB activation are initiated in response to short-wavelength ultraviolet light (UV) or DNA damage [79, 136-141]. As an example, the NF-κB response to DNA damage will be described. NF-κB activation upon DNA-damage is still poorly understood. A series of posttranslational events, including sumoylation, ubiquitination, phosphorylation and nuclear-cytoplasmic shuttling of NEMO appear critical [142], but the order and which molecules fine-tuning these events need to be ravealed. A nuclear poly (ADP-ribose) polymerase 1 (PARP-1), ataxia telangiectasia mutated (ATM)-kinase, protein inhibitor of activated STAT4 (PIASy) and NEMO signalosome are defined as critical nuclear components of the DNA damage-induced NF-κB signaling pathway [143-145]. The required cytoplasmic proteins involve ATM that is translocated to the cytoplasm, TRAF6 and/or ELKS (protein rich in glutamate, leucine, lysine and serine), as well as the E2 ligase Ubc13 and members of the inhibitor of apoptosis (IAP) family [146, 147]. The latter act as E3 ligases conducting assembly of Lys63-polyUb scaffolds thereby facilitating TAK1-TAB2/3-mediated activation of a functional IKK complex [144, 145]. In addition, linear Ub chains assembled on NEMO by LUBAC were shown to be essential for the DNA damage induced NF-κB activation [148] (Fig. 1.6B).

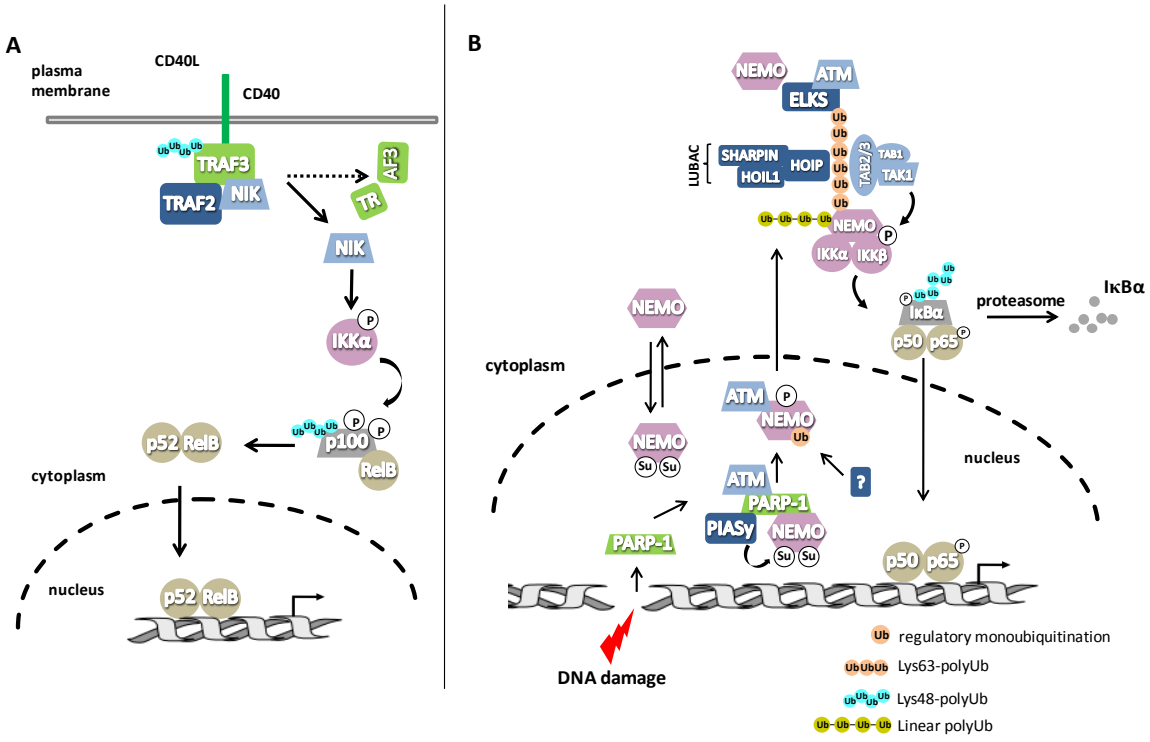



**Figure 1.6 (A) Non-canonical NF-κB signaling pathway: CD40 ligand-induced activation.** Binding of the CD40 leads to the activation of NIK which phosphorylates IKKα. Activated IKKα phosphorylates the precursor p100 leading to formation of the mature p52 and nuclear translocation of RelB/p52 dimer. Scheme modified from [132]. **(B) Alternative signaling pathway to NF-κB: DNA damage.** Following DNA damage, nuclear events trigger activation of the ATM kinase and PTMs of NEMO. These nuclear events are transduced to cytoplasmic signaling complexes that mediate the activation of TAK1 and ultimately IKK. Scheme modified form [147].

## 1.4 IκB-independent regulation of NF-κB activity by PTMs of p65

All NF-κB DNA-binding subunits undergo PTMs [149-151]. The best studied family member in this respect is the p65 subunit. An additional layer of NF-κB regulation is provided by covalent modifications of the NF-κB subunits that alter the ability of the NF-κB dimers to bind DNA, recruit coactivators to the enhancer region and interact with IκBα, thus influencing the amplitude and duration of the NF-κB response. Many modifications are likely to differ between cell types and the nature of the NF-κB-inducing stimulus and effects of the p65 site-specific modifications spans from transcriptional activation to the complete repression of certain genes. p65 phosphorylation at different sites serves as an integrator for multiple incoming signals, which can further control subsequent p65 modifications. The PTMs of p65 and their functional consequences will be described in detail in the following sections. Figure 1.7 shows a schematic presentation of modifications and involved enzymes.

### 1.4.1 NF-κB regulation by p65 phosphorylation and dephosphorylation

The PhosphoSitePlus database http://www.phosphosite.org lists eighteen identified p65 phosphorylation sites. Twelve of these sites have also been characterized for their functional relevance (Fig. 1.7).

#### 1.4.1.1 NF-κB p65 phosphorylation

Ser276 is one of the well characterized p65 phosphorylation sites. It is located in the DD and has an influence on p65 transcriptional activation [152], intracellular localization [153], protein-protein interactions [154, 155] and protein stability [156]. In response to LPS, p65 is phosphorylated at Ser276 by PKAc (catalytic subunit of protein kinase A). The stimulus-induced degradation of IκBα is a prerequisite for the activation of PKAc and the subsequent p65 Ser276 phosphorylation occurs exclusively within the cytoplasm [157]. Phosphorylation at Ser276 induces a conformational change that allows binding to the CBP (CREB-[c-AMP-response element-binding protein -binding protein) protein [154]. Serine 276 is also targeted by the kinase MSK-1 (mitogen- and stress-activated protein kinase-1) upon TNF stimulation. This kinase directly associates with p65 in a strictly stimulus-dependent manner and phosphorylates p65 at Ser276 in the nucleus [152, 158]. Reconstitution experiments and the analysis of p65 Ser276 to alanine knock-in mice revealed that effects of p65 phosphorylation at Ser276 are rather promoter specific [152, 159-162]. Nowak *et al*. showed that p65 Ser276 phosphorylation is required for activation of a subset of genes. Upon TNF stimulation, Ser276 phosphorylated p65 binds to P-TEFb (positive transcription elongation factor b), which controls the RNA polymerase II (RNA Pol II) recruitment and activation of transcriptional elongation of distinct genes. On the other hand, some of genes like *IκBα* are preloaded with the RNA Pol II in the absence of stimulation. Even though stimulation leads to the p65 Ser276 phosphorylation and P-TEFb recruitment to the *IκBα* gene, this complex is not required for *IκBα* promoter activation [161].

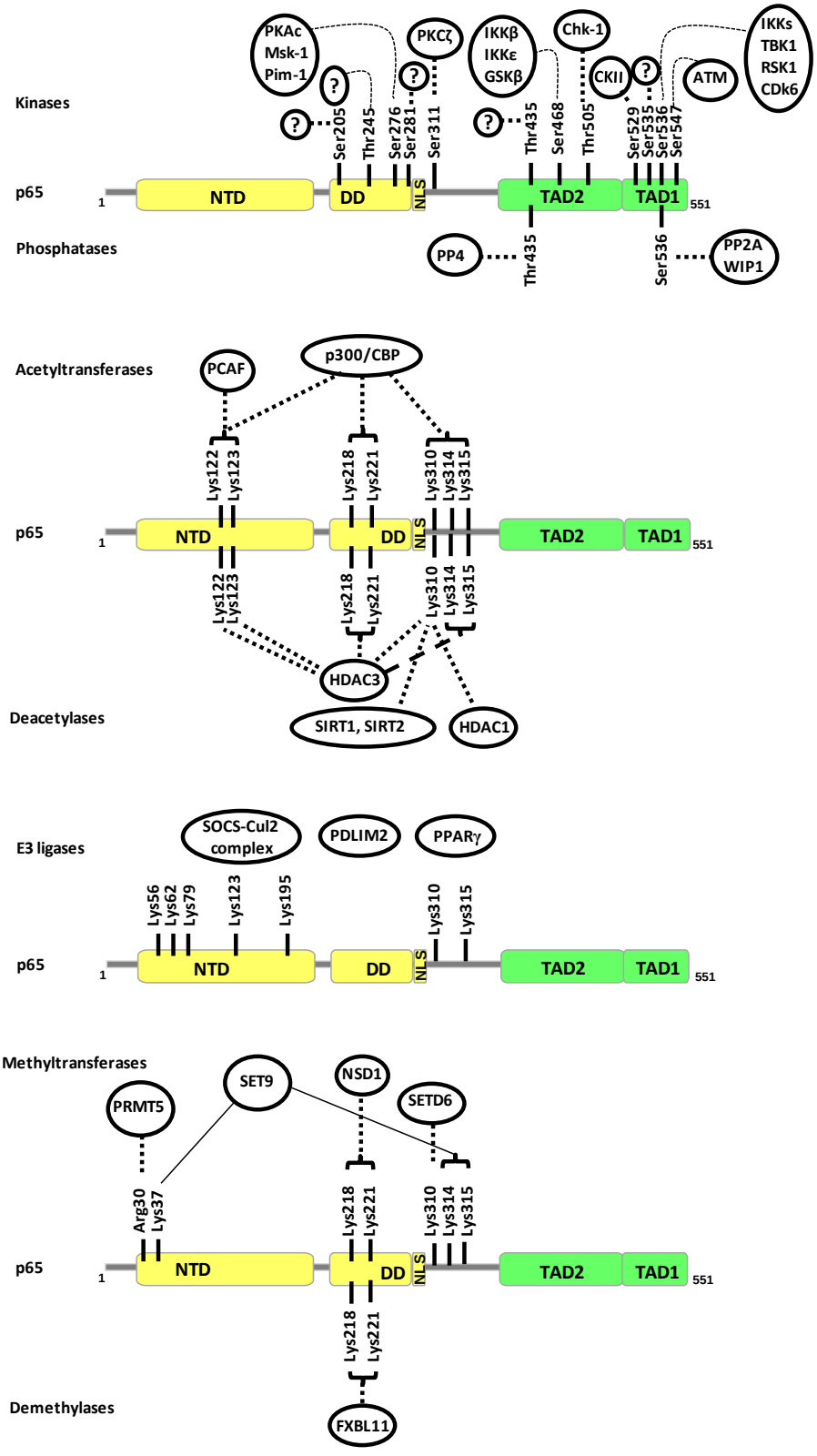



**Figure 1.7 PTMs of p65.** Schematic representation of p65 phosphorylation, acetylation, ubiquitination and methylation sites. The modifying enzymes that catalyze addition and removal of covalent modifications are indicated. ?- not identified, the scheme was modified from [163].

The serine/threonine kinase Pim-1 (proviral integration site for the Moloney-murine leukemia virus-1) was also identified as a p65 kinase that mediates phosphorylation of Ser276 and subsequent activation of p65. This study suggested that Ser276 phosphorylation protects p65 from proteasomal degradation [156].

In response to TNF, p65 is phosphorylated at Thr245 by an unknown kinase [164]. Phosphorylation at this site creates a phospho-Thr/Pro motif which is a target of another PTM catalysed by the enzyme peptidyl-prolyl isomerase Pin-1 (peptidyl-prolyl cis-trans isomerase NIMA-interacting-1). Pin-1 isomerisation disrupts p65 association with IκBα and induces its translocation to the nucleus. This Pin-1-dependent mechanism also protects p65 from SOCS-1 (suppressor of cytokine signaling-1)-mediated ubiquitination and subsequent proteolysis. The effect of p65 isomerization on DNA-binding or transcriptional activity is still unknown.

PKCζ (protein kinase C, zeta) phosphorylates p65 at Ser311 in a TNF-dependent manner. Phosphorylaytion at this site enhances the recruitment of CBP to the *Il-6* promoter, thus enhancing the transcriptional activity of NF-κB [165]. On the other hand, a study using a site-directed mutagenesis approach revealed that Ser311 phosphorylation is not essential for p65 transcriptional activity, but it is required for transcription of a minority of NF-κB genes [162].

Residues Ser468 and Ser536 are the most studied C-terminal phosphosites. Phosphorylation of p65 at Ser536 is mediated by different kinases: IKKα, IKKβ, TBK1 (TANK (TRAF family member-associated NF-κB activator)-binding kinase 1), CDK6 (cyclin dependent kinase 6) and RSK-1 (ribosomal protein S6 kinase 1) and is induced by various agents, such as TNF, IL-1, T-cell costimulation, human T lymphotrophic virus-1 (HTLV-1)-encoded Tax protein and cytotoxic agents [166-173]. Upon TNF treatment or T-cell costimulation, NF-κB phosphorylated at p65 Ser536 has been mainly detected in the cytosol, in particular in the area surrounding the nucleus. Those findings implicate the role of Ser536 phosphorylated p65 in the control of NF-κB nuclear import kinetics and allows a fine tuning of the NF-κB mediated transcriptional response [166, 168]. In contrast, RSK-1- and TBK-1-mediated phosphorylation of Ser536 decreases the affinity between p65 and IκBα and reduces IκBα-mediated nuclear export of NF-κB, thereby promoting the binding and action of NF-κB on cognate κB enhancers [170, 172]. Functional characterization of

phospho-Ser536 in *Il-8* transcription revealed that phosphorylation at this site modulates the balance between binding of TAFII31 (TATA-binding-protein-associated factor II31), a component of the general TF IID complex, and the corepressor AES (amino enhancer of split) [170]. The IKKα-mediated phosphorylation of p65 at Ser536 and of corepressor SMRT (silencing mediator for retinoic acid receptor and thyroid hormone receptor) at Ser2410 prevents repressor complex recruitment and its association to the NF-κB promoter. This dual phosphorylation thus allows the loading of p300 to the promoter and subsequent p65 acetylation at Ser310, which is required for full NF-κB transcriptional activity [174, 175]. On the other hand, IKKα-dependent phosphorylation of p65 at Ser536 in macrophages results in accelerated turnover of this subunit, thereby facilitating their removal from the promoters and terminating NF-κB-mediated gene induction [176].

Phosphorylation of Ser536 and Ser468 is not mediated by same kinases, except IKKβ which has been found to phosphorylates both sites after the T cell costimulation [166, 177]. Ser468 is the target of at least three protein kinases: IKKβ, IKKε and GSK-3β (glycogen-synthase kinase-3beta) [177-179]. Loss-of-function experiments using small-hairpin RNA (shRNA)-mediated IKKε knock-down showed that TNF-induced Ser536 phosphorylation was independent from IKKε, while Ser468 phosphorylation was largely impaired in the absence of this kinase [166]. Serine 468 phosphorylation is described as a nuclear event and NF-κB phosphorylated at p65 Ser468 is predominantly located within the nucleus [166, 179, 180]. On the other hand, IKKβ-mediated phosphorylation at this site was reported to be cytoplasmic while the NF-κB is still bound to IκBα [177]. Ser468 phosphorylation has been described as both stimulating and inhibiting p65 transactivation [166, 177, 179]. Different outcomes could be explained by a NF-κB barcode hypothesis, according to which phosphorylation at Ser468 alone or in combination with other PTMs could generate distinct patterns that function to direct transcription in a target gene-specific fashion [166]. Also, phosphorylation by GSK-3β enables p65 to recruit Nurr1 (nuclear receptor related-1 protein) to the NF-κB on the target gene promoters. This is followed by recruitment of the CoREST (corepressor for REST (RE1 silencing TF)) corepressor complex, resulting in clearance of p65 and inhibition of basal NF-κB activity [178, 181]. Phosphorylation of Ser468 by IKKβ or IKKε in response to TNF or IL-1 attenuates the activity of NF-κB by enhancing the binding of a COMMD1 (copper metabolism MURR1 domain-containing protein 1)-containing E3 ligase complex, resulting in Lys48-linked

ubiquitination and target gene-specific proteasomal degradation of NF-κB [182, 183]. In response to proapoptotic stimuli, COMMD1 also mediates p65 ubiquitination through interaction with p65. This acts as a signal for nucleolar translocation of the p65, but this recruitment of the COMMD1-containing E3 ligase complex is independent from p65 Ser468 phosphorylation [184].

TNF-induced Thr435 phosphorylation by an unknown kinase disrupts the interaction of p65 with HDAC1 (histone deacetylase 1) and enhances histone acetylation associated with decreased recruitment of HDAC1 on target-gene promoters [185]. On the other hand, tumor suppressor ARF (alternate reading frame) or cisplatin-induced phosphorylation of Thr505 by Chk1 (checkpoint kinase 1) inhibits p65 transactivation. Thr505 phosphorylation increases p65 association with HDAC1, resulting in transcriptional repression of some NF-κB target genes, like Bcl-xL (B-cell lymphoma-extra large) [186, 187]. In response to genotoxic stimuli, activated ATM kinase directly phosphorylates p65 at Ser547 resulting in decreased expression of a specific set of inflammatory NF-κB target genes by a mechanism involving HDAC recruitment [188]. Phosphorylation at Ser529 only moderately enhances the NF-κB-dependent transcription [189, 190], while phosphorylation of Ser535 mediated by CaMKIV (calmodulin-dependent protein kinase IV) increases NF-κB-dependent transcription [191].

#### 1.4.1.2 Dephosphorylation

Dephosporylation is an important step in re-establishing the normal responsiveness of NF-κB. Protein phosphatase 2A (PP2A) interacts with p65 and directly dephosphorylates p65 under basal conditions [192]. A systematic RNAi (RNA interference) screen of phosphatases also identified PPA2 as a phosphatase responsible for Ser536 and Ser276 dephosphorylation, leading to inhibition of NF-κB transcriptional activity [193]. WIP1 (wild-type p53-induced phosphatase) was identified as another Ser536 phosphatase, reducing the interaction between p65 and p300 and thereby target gene transcription [194]. The function of NF-κB is also regulated through dephosphorylation of p65 at Thr435 by protein phosphatase 4 (PPA4) in response to cisplatin treatment [195].

### 1.4.2 NF-κB regulation by p65 acetylation and deacetylation

Acetylation of different lysines leads to different effects on p65 DNA binding, transcriptional activity, interaction with IκBα proteins and subcellular localization [94, 196, 197]. Acetylation of Lys122 and Lys123 reduces p65 binding to the κB element and seems to negatively regulate NF-κB mediated transcription [197]. Acetylation at Lys221 enhances p65 binding to DNA and together with acetylated Lys218 impairs the p65 association with newly synthesized IκBα, preventing the relocation of the NF-κB complex to the cytoplasm. The same study emphasizes the positive role of Lys310 acetylation on the transactivation potency of p65 [198]. TNF-induced p65 acetylation at Lys314 and Lys315 by p300 neither affects NF-κB shuttling, DNA binding nor the induction of anti-apoptotic genes, but differentially regulates the expression of specific sets of NF-κB target genes [199, 200]. NF-κB p65 knockout mouse embryonic fibroblasts (MEFs) reconstituted with wild type p65 or its acetylation-mimicking mutant forms where five acetylation acceptor sites Lys122, Lys123, Lys314, Lys315 and Lys310 were changed to glutamine where tested for their capacity to regulate gene expression. These showed that acetylation inhibits the expression of most IL-1-induced p65 target genes such as *Vcam1, Il-6, Lamb3.* This finding indicates that the effect of p65 acetylation is rather gene specific and that acetylation should not be considered as a PTM that amplifies the activation signal [201].

The histone deacetylases HDAC1, HDAC2, HDAC3, SIRT1 and SIRT2 deacetylate p65 and regulate functions of NF-κB [197, 202-206]. Early studies showed that deacetylation of p65 by HDAC3 inhibits the transcriptional activity of NF-κB and also enhance the nuclear export of the NF-κB-IκBα complex by promoting the interaction between NF-κB and IκBα [198, 207]. In contrast to this, a recent study showed that HDAC3 functions as a coactivator by binding to p65 and removing the inhibitory p65 acetylations at Lys122, Lys123, Lys134 and Lys135 [197, 206]. Deacetylation of Lys310, mediated by SIRT1 and SIRT2, inhibits the transcriptional activity of NF-κB and sensitizes cells for TNF-induced apoptosis [204, 205].

### 1.4.3 NF-κB regulation by p65 ubiquitination

Seven Ub acceptor sites in the N-terminus of p65 were identified by mass spectrometry (MS): Lys56, Lys62, Lys79, Lys123, Lys195, Lys310 and Lys315. Additionally, the MS data confirmed the p65 modification by Lys48-polyUb chains and also showed that this

subunit can be modified by Lys29-, Lys33- and Lys63-polyUb chains [202]. The covalent conjugation of Ub to cellular proteins regulates various cell processes. Ubiquitination of p65 mainly favours the termination of NF-κB activity by promoting the degradation of a fraction of DNA-bound and active p65 in a gene-specific manner [208, 209]. NF-κB p65 ubiquitination is mediated by a SOCS-Cul2 (cullin-2) containing E3 ligase complex in which SOCS1 functions as a substrate receptor. This complex contains two regulators of SOCS1 for the ubiquitination of p65-COMMD1 and the histone acetyltransferase GCN5 (general control nonderepressible 5). COMMD1, first identified as an NF-κB inhibitor, provides ubiquitination and degradation of nuclear p65 by stabilizing the interaction between SOCS1 and p65. Phosphorylation at Ser468 facilitates p65 ubiquitination by promoting the interaction with GCN5, which mediates p65 interaction with the COMMD1/Cul2-containing E3 ligase complex [182, 183, 210]. Recent studies showed that upon IL-1 stimulation, SOCS1 exclusively binds to p65 within the nucleus and has access to p65 only when it is bound to DNA, since the DNA binding mutant did not interact with SOCS1 [211]. In response to aspirin, COMMD1-mediated p65 ubiquitination targets p65 for nucleolar translocation [212]. PDLIM2 (PDZ and LIM domain 2)-mediated p65 ubiquitination shuttles nuclear p65 into PML (promyelocytic leukemia protein) nuclear bodies where it is degraded by the proteasome .

### 1.4.4 NF-κB regulation by p65 methylation

Lysine methylation has recently emerged as another important modification which regulates the transcriptional activity of NF-κB depending on the position of the methylation site. The SET (suppressor of variegation-enhancer of zeste-trithorax) domain histone lysine methyltransferase Set9 has been identified as a p65 methyltransferase. In response to TNF, Set9 interacts with p65 and monomethylates p65 at Lys314 and Lys315. This dual monomethylation induces the proteasome-mediated degradation of a promoter-associated p65 and terminates NF-κB activity [213]. On the other hand, Set9-mediated p65 monomethylation at Lys37 appears to be important for the activation of a subset of NF-κB target genes by stabilizing the NF-κB binding to DNA [214]. The kinetics of p65 methylation by Set9 in response to TNF showed that maximal Lys37 methylation appears at 30 min [214]**,** whereas Lys314 and Lys315 modification appears after 60 min [213], implying that Set9 sequentially methylates different lysines during the course of NF-κB activation which exert different effects [214]. The NSD1 (nuclear receptor-binding SET

domain protein 1) methyltransferase methylates p65 at Lys218 and Lys221. Methylation of p65 by NSD1 enhances the transcriptional activity of NF-κB and expression of NF-κB target genes. Demethylation of Lys218 and Lys221 by FBXL11 (F-box and leucine-rich repeat protein 11) negatively regulates the transcriptional activity of NF-κB [215]. Recently, Hur *et al.* reported that glioma-expressed antigen-2 PHF20 (PHD finger protein 20) interacts with p65 by recognizing methylated Lys218 and Lys221 [216]. This methylation-dependent interaction between PHF20 and p65 leads to persistent NF-κB phosphorylation and limits the recruitment of protein phosphatase PP2A to p65. A screen of 40 candidate p65 methyltransferases identified SETD6 (SET domain-containing protein 6) as an enzyme that monomethylates chromatin-associated NF-κB subunit p65 at Ser310. Monomethylation of nuclear p65 at Lys310 attenuates NF-κB signaling through recruitment of another methyltransferase GLP (G9A-like protein). Under basal conditions GLP promotes a repressed chromatin state at p65 target genes by methylation of histone H3 at Lys9. SETD6 p65 Lys310 monomethylation occurs in the absence of stimulation and is functionally suppressed by TNF-induced phosphorylation of p65 at the neighboring Ser311 [217, 218]. Very recently it has been found that the p65 subunit is dimethylated on Arg30 by PRMT5 (protein arginine methyltransferase 5) in response to IL-1. A microarray analysis using HEK-293IL-1R cells overexpressing wild type p65 or the Arg30 to alanine mutant p65 protein showed that p65 Arg30 dimethylation is the prerequisite for activation of 75% of all p65-dependent genes. Structural data suggest that dimethylation at Arg30 increases the ability of p65 to bind DNA and thus affects gene expression [219].

## 1.5 NF-κB regulation by molecular chaperones

### 1.5.1 Molecular chaperones and protein folding

The biological functions of proteins are governed by their three-dimensional (3D) folding. Following synthesis on ribosomes as linear sequences of amino acids, the vast majority of proteins must fold into well-defined 3D structures (their native state) to attain functionality. The folded 3D structures of most proteins represent a compromise between thermodynamic stability and the conformational flexibility required for function. Proteins are often marginally stable under the physiological conditions inside the cell and thus susceptible for misfolding and aggregation [220]. In addition, a substantial fraction of proteins in eukaryotic cells (~30%) are classified as intrinsically unstructured and contain

regions thought to adopt ordered structure only upon interaction with binding partners [221]. Because of a great variety of possible conformations that protein chain can adopt, folding reactions are highly complex and heterogeneous and rely on the cooperation of multiple weak, noncovalent interactions. Among these, hydrophobic forces are critical in driving chain collapse and the burial of nonpolar amino acid residues within the interior of the folded protein. In particular, proteins with complex structure may expose hydrophobic amino acid residues to the solvent during folding, rendering them susceptible to nonnative interactions that lead to aggregation [220]. To counteract these nonnative interactions, cells have a network of molecular chaperones that assist in *de novo* folding and also maintain pre-existing proteins in their native states. By definition, a molecular chaperone is any protein that interacts with and aids in the folding or assembly of another protein without being part of its final structure. Many chaperones are members of the heat shock protein (Hsp) families. Synthesis of the Hsp is induced under the stress conditions, like heat shock or oxidative stress, when a subset of cellular proteins are structurally destabilized [222].

Mammalian Hsps have been classified mainly in four families according to their molecular weight: Hsp90, Hsp70, Hsp60 and small (15-30 kDa) Hsps that include Hsp27. Members of the Hsp families are either constitutively or inducibly expressed. They are present in different cellular compartments, including cytosol, mitochondria, endoplasmic reticulum and the nucleus [223]. Besides their role in proper protein folding, stress-inducible Hsps exhibit their cytoprotective effect by interfering with apoptosis at different stages. This function is mediated through associations with key effectors of anti-apoptotic proteins. As an example, Hsp90 and Hsp70 binds to the apoptotic protease activating factor-1 (Apaf-1), thereby inhibiting caspase activation and apoptotic cell death [224]. Additionally, Hsps contribute to cell survival through stabilisation or CHIP (carboxyl-terminus of Hsp70 interacting protein)-mediated proteasomal degradation of selected proteins under stress conditions [225-227].

### 1.5.2 Heat shock protein 90

Hsp90 is a highly abundant chaperone protein expressed in all eukaryotic cells. In humans the most prominent members are the Hsp90 alpha (Hsp90α) and Hsp90 beta (Hsp90β) isoforms. They are expressed by two different genes and share 86% of homology. Hsp90 functions as a part of a multichaperone complex via association with a variety of co-

chaperones and client proteins that rely on the complex for maturation and stability. Hsp90 exists as a homodimer and contains three relevant domains (Fig. 1.8). The N-terminal domain contains the ATP (adenosine triphosphate)-binding pocket, which is responsible for the ATPase activity. A charged middle linker region has a high affinity for co-chaperones (Cdc37, p23, Aha1) and client proteins. The C-terminus contains the DD that harbors the tetratricopeptide repeat (TRP)-binding motif. This motif mediates the interaction of Hsp90 with TRP-containing co-chaperones such as HOP (Hsp organizing protein), a protein responsible for the proper ATPase function of Hsp90 [223, 228].

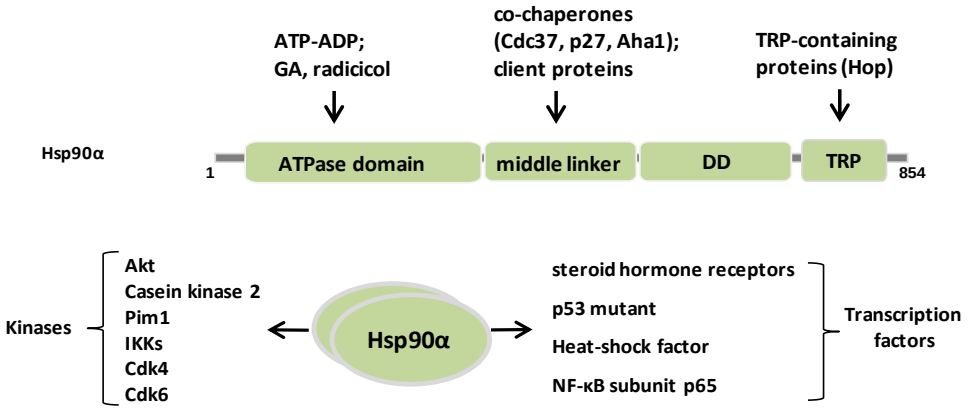



**Figure 1.8 Schematic representations of Hsp90α and its client proteins.** Upper: Hsp90α protein with the indicated functional domains and regions responsible for interaction with the co-chaperones, TRP-containing proteins and inhibitors. Lower: Hsp90α is represented as a dimer with some indicated Hsp90 client partners. Abbreviations: ADP (adenosine diphosphate).

In eukaryotes, many signal proteins are transferred from Hsp70 to the ATP-dependent Hsp90 chaperone system to further take care of the folding and conformational regulation [229]. Various proteins implicated in almost all cellular processes are regulated by Hsp90, most of them being involved in signal transduction and functioning as TFs and kinases [230, 231] (Fig. 1.8). Therefore, Hsp90 affects key cellular processes, including cell cycle progression, steroid signaling, protein trafficking, protein secretion, the immune response and the heat shock response. Pharmacological inhibition of Hsp90 with geldanamycin (GA) or its derivatives results in an unfolded or partially folded state of many kinases, reducing their activity and/or increasing their degradation [232]. Hsp90 inhibitors are in clinical development for cancer therapy [223].

### 1.5.3 Heat shock protein 70

The Hsp70 family consists of several highly related members. Among them the highly stress-inducible Hsp70/Hsp72 proteins and the constitutively expressed Hsc70/Hsp73 proteins are best studied [233]. The Hsp70s are an important part of cells machinery for the protein folding and help to protect cells from stress [234]. Hsp70 contains two distinct functional regions: the N-terminal ATPase domain and the C-terminal peptide binding domain with TRP-binding motif (Fig. 1.9). The peptide-binding domain is responsible for substrate binding and refolding of the Hsp70 client proteins. The ATPase domain, in turn, facilitates the release of the client protein after ATP hydrolysis. The C-terminal TRP domain mediates interaction with the TRP-containing proteins, such as co-chaperones Hop (Hsp70-Hsp90 organizing protein) and CHIP [223, 235].

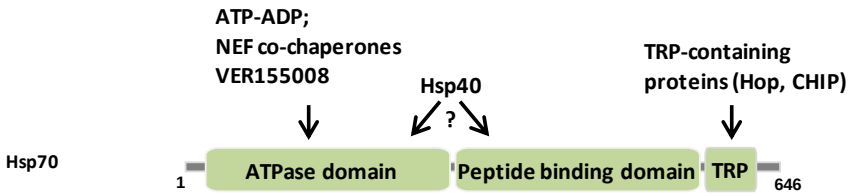



**Figure 1.9 Schematic representation of Hsp70.** The Hsp70 protein is shown with the indicated functional domains and regions responsible for interaction with the co-chaperones, TRP-containing proteins and inhibitor VER155008 (VER). It is still not known whether the Hsp40 make a contact with ATPase domain or peptide-binding domain. Abbreviations: NEF (nucleotide exchange factor).

The Hsp70 chaperone function is regulated by distinct co-chaperones. The J-domain co-chaperone, also known as Hsp40, accelerates hydrolysis of ATP to ADP and mediates interaction between the substrate and Hsp70. Hsp40 also can interact directly with unfolded polypeptides and recruits Hsp70 when needed [236, 237]. The NEF co-chaperones (e.g. Bag-1, Hsp110, HspBP1) catalyze the release of ADP required for completion of the Hsp70 ATPase cycle. TRP-containing co-chaperones (Hop, CHIP) are essential for combinatorial assembly of Hsp70 and Hsp90 complexes. Additionally, CHIP as a Ub ligase, ubiquitinates Hsp/Hsc70 substrates promoting their degradation by the proteasome [228, 238]. Degradation-independent functions of CHIP are also observed [239, 240]. Hsp70 also regulates the conformation of TFs and various kinases [241-243]. Constitutively high expression of Hsp70 is essential for the survival of most cancer cells

[244]. In experimental models, Hsp70 overexpression increases the tumorigenicity of cancer cells while Hsp70 down-regulation strongly decreases tumorigenicity [245]. Neutralizing Hsp70 is therefore an attractive strategy for anticancer therapy. Recently it has been shown that Hsp70 inhibitor VER significantly reduces the division of myeloma cells with limited effects on normal blood cells [246]. Furthermore, VER in combination with the GA analogue 17-AAG (17-N-Allylamino-17-demethoxygeldanamycin) blocks myeloma cell division more effectively than either inhibitor alone. Also, treatment with both VER and 17-AAG induced apoptosis in HCT116 colon carcinoma cells [247].

### 1.5.4 Hsp proteins and NF-κB signaling

Hsp90 and its co-chaperone Cdc37 are constitutively associated with the IKK complex. Hsp90 is recruited to the IKK complex via direct interaction between co-chaperone Cdc37 and the catalytic regions of IKKα and IKKβ [248]. The Hsp90 inhibitor GA inhibits TNF-induced activation of IKK and NF-κB. The inhibitor also prevented the recruitment of the IKK complex to the TNFR1, indicating a role of Hsp90/Cdc37 in activation and trafficking of IKKs from the cytoplasm to the membrane. Another study showed that Hsp90 is also required for stabilization or folding of newly synthesized IKKα and IKKβ proteins during translation [249]. Hsp90 also associates with other kinases involved in the NF-κB pathway: RIP1, MEKK3 (mitogen-activated protein kinase kinase kinase 3), MEKK1, NIK, Akt, TBK1 [250-253]. Functional inhibition of Hsp90 by GA efficiently disrupts its direct interaction with NIK, resulting in ubiquitination-proteasome system-independent NIK degradation and subsequent blockage of p100 processing. Inhibition with GA does not affect *Nik* mRNA transcription and translation, which indicates that Hsp90 is involved in the folding/stabilization of NIK protein [254]. It has been shown that Hsp70 directly interacts with NEMO through NEMO´s CC domain, precluding NEMO oligomerisation and the assembly of the IKK complex [255]. Overexpression of the co-chaperone Hsp27 stimulates the ubiquitination-mediated proteasomal degradation of phosphorylated IκBα and thereby increases the activity of NF-κB. Hsp27 is a Ub-binding protein and under stress conditions it interacts with the 26S proteasome and favors the degradation of ubiquitinated IκBα [256]. The major stress protein Hsp70 interacts with p65, p50 and c-Rel, where the interaction of p65 and p50 with Hsp70 is constitutive and further enhanced upon stimulation with phorbol myristoylacetate (PMA) after the exposure to heat shock [257]. Recently, MS analysis of extracts from neurons revealed an interaction between the

NF-κB subunit p65 and Hsp90α and Hsp90β and identified Hsc70 as a novel interaction partner of p65 [242]. Mortalin, a mitochondrial Hsp70, has been also identified as a p65 interacting partner and mediates the mitochondrial localization of p65 [258].

## 1.6 Aims of the study

PTMs provide an additional layer of regulation for the activity of TFs. Previous data in the lab have shown that mutation of a single phosphorylation site in p65 (Ser468) largely prevented association with COMMD1 and further members of COMMD1-containig E3 ligase complex, suggesting that this mutation alters the overall conformation of NF-κB p65 [182]. Also, as literature data raised the possibility that p65 undergoes a stimulus-induced conformational change [259], this study aimed to address the question whether p65 conformation is regulated in a stimulus-dependent fashion. For that purpose assays that allow the determination of p65 conformation *in vitro* should be developed. To examine the impact of a single phosphosite on p65 conformation, point mutants of phosphorylated residues should be created. A possible contribution of chaperones on p65 folding and activity should be investigated. As several studies suggested a p65 conformational change upon binding to a κB site [259, 260], the possible contribution of p65 DNA-binding should be analysed. To address this question, p65 DNA-binding mutants should be produced and used to reconstitute p65-deficient MEFs. These mutants should also allow investigations of non-genomic functions of the p65 protein.

# 2. Materials and Methods

## 2.1 Materials

### 2.1.1 Antibodies

| Primary antibody | Species | Supplier |
|---|---|---|
| anti-β-Actin | rabbit, polyclonal antibody (pAb) | Abcam |
| anti-c-Rel | rabbit, pAb | Santa Cruz Biotech |
| anti-Flag (M2) | mouse, monoclonal antibody (mAb) | Sigma |
| anti-Gal4 | rabbit, mAb | AG Schmitz |
| anti-GFP | mouse, mAb | Roche |
| anti-HA (3F10) | rat, mAb | Roche |
| anti-HDAC1 (H11) | mouse, mAb | Santa Cruz Biotech |
| anti-Hsp70/72 (C92F3A-5) | mouse, mAb | Enzo Life Science |
| anti-IκBα (C-21) | rabbit, pAb | Santa Cruz Biotech |
| anti-IκBβ (C-20) | rabbit, pAb | Santa Cruz Biotech |
| anti-IκBε (M121) | rabbit, pAb | Santa Cruz Biotech |
| anti-Acetyl-Lys | rabbit, pAb | Cell Signaling Tech |
| anti-p50 (C-19) | goat, pAb | Santa Cruz Biotech |
| anti-p50 (E-10) | mouse, mAb | Santa Cruz Biotech |
| anti-p52 (c-5) | mouse, mAb | Santa Cruz Biotech |
| anti-p65 (A) X | rabbit, pAb | Santa Cruz Biotech |
| anti-p65 (F-6) | mouse, mAb | Santa Cruz Biotech |
| anti-p65 (C-20) | rabbit, pAb | Santa Cruz Biotech |
| anti-phospho-IκBα (Ser 32/36) (5A5) | mouse, mAb | Cell Signaling Tech |
| anti-phospho-p65 (Ser276) | rabbit, pAb | Cell Signaling Tech |
| anti-phospho-p65 (Ser468) | rabbit, pAb | Cell Signaling Tech |
| anti-phospho-p65 (Ser 536) | rabbit, pAb | Cell Signaling Tech |
| anti-RelB (C-19) | rabbit, pAb | Santa Cruz Biotech |
| anti-Tubulin (Tub2.1) | mouse, mAb | Sigma |
| anti-Ub (P4D1) | mouse, mAb | Cell Signaling Tech |

| Primary antibodies used for the p65 conformational study | Species | Supplier (catalog number) |
|---|---|---|
| anti-p65 | rabbit, pAb | Imgenex (IMG-5915A) |
| anti-p65 | rabbit, pAb | Imgenex (IMG-5098) |
| anti-p65 (6H7) | mouse, mAb | Abcam (ab91626) |
| anti-p65 | rabbit, pAb | Imgenex (IMG-5238) |
| anti-p65 | rabbit, pAb | Imgenex (IMG-6066A) |
| anti-p65 (EP2161Y) | rabbit, mAb | Abcam (ab76311) |
| anti-p65 (112A1021) | mouse, mAb | Imgenex (IMG-150A) |

| Secondary antibody | Conjugated to | Supplier |
|---|---|---|
| goat-anti-mouse IgG | Horseradish peroxidase | Dianova |
| goat-anti-rabbit IgG | Horseradish peroxidase | Dianova |
| goat-anti-rabbit IgG | Cy$^{TM}$3 | Dianova |
| goat-anti-rat IgG | Horseradish peroxidase | Dianova |

### 2.1.2 Bacterial strains

| Strain | Genotype | Supplier |
|---|---|---|
| BL21 | F- *omp*T *hsd*SB(rB-, mB-) *gal dcm* | Novagene |
| BL21pLysS | F-*omp*T *hsd*SB(rB-, mB-)*galdcm* pLysS (CamR) | Invitrogen |
| GM2163 | F-*ara-14 leuB6 fhuA31 lacY1 tsx78 glnV44 galK2 galT22 mcrA dcm-6 hisG4 rfbD1 rpsL136 dam-13::*Tn*9*(CamR) *xylA5 mtl-1 thi-1 mcrB1 hsdR2* | New England Biolabs |
| Top10 | F-*mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lacZ*ΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*araleu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G | Invitrogen |

### 2.1.3 Eukaryotic cell lines

| Strain | Cell type |
|---|---|
| HEK-293 | Human embryonic kidney cells |

| | |
|---|---|
| HEK-293T | HEK 293 cells expressing the large T antigen of the SV40 virus |
| HEK-293IL-1R | Human embryonic kidney cells expressing IL-1 receptor |
| HeLa | Human cervical carcinoma cells |
| MEFs | Mouse embryonic fibroblasts |
| p65 $^{-/-}$ MEFs | MEFs lacking the p65 gene |
| mRelAθ | p65 knock-out MEFs, reconstituted with the empty EGF9 vector |
| mRelA *wt* | p65 knock out MEFs, reconstituted with the wild type mouse p65 |
| mRelA E39I | p65 knock out MEFs, reconstituted with the mouse p65 E39I mutant |
| mRelA E39I/R302E | p65 knock out MEFs, reconstituted with the mouse p65 double E39I/R302E mutant |
| mRelA S468A | p65 knock out MEFs, reconstituted with the mouse p65 S468R mutant |
| mRelA S536A | p65 knock out MEFs, reconstituted with the mouse p65 S536A mutant |

### 2.1.4 Plasmids

#### 2.1.4.1 Expression plasmids

| cDNA | Vector | Epitope-tag | Supplier |
|---|---|---|---|
| CBP | | YFP | L. de la Vega, Giessen |
| Gal4 | pAB | None | M.L. Schmitz, Giessen |
| Hsc70 | pCMV | Flag | J. Young, Quebec |
| Hsp70 | pCMV | Flag | B. Song, Davis, CA |
| Hsp70 | pCMV | HA | B. Song, Davis, CA |
| Hsp90α | pCDNA3 | Flag | B. Song, Davis, CA |
| MYBBP1a | pCMV | Flag | M.O. Hottiger, Zurich |
| p50 | pCMV | Flag | M.L. Schmitz, Giessen |
| p65 | pEF | HA | I. Mattioli, Bern |
| p65 | pCDNA3 | HA | T. Wittwer, Giessen |
| p65 S276A | pCDNA3 | HA | M. Milanovic, Giessen |
| p65 S276E | pCDNA3 | HA | M. Milanovic, Giessen |
| p65 S468A | pEFpuro | HA | H. Geng, Giessen |
| p65 S468E | pEFpuro | HA | H. Geng, Giessen |

| | | | |
|---|---|---|---|
| p65 S536A | pEFpuro | HA | H. Geng, Giessen |
| p65 S536E | pEFpuro | HA | H. Geng, Giessen |
| p65 | pEGFP | GFP | M. Kracht, Hannover |
| p65 | pAB | Gal4 | M.L. Schmitz, Giessen |
| p65 | pCMV | Flag | M.L Schmitz, Giessen |
| p300 | pCDNA3 | HA | L. de la Vega, Giessen |
| Ub | pCMV | His-HA | R. Bear, New York |

*YFP (yellow fluorescent protein); *HA (hemagglutinin); *GFP (green fluorescent protein); * His (histidine)

### 2.1.4.2 Plasmids for lentiviral production

| cDNA | Vector | Epitope-tag | Supplier |
|---|---|---|---|
| θ | FG9 EF1α Puro | None | E. Burstein, Dallas |
| mp65 wt | FG9 EF1α Puro | HA | E. Burstein, Dallas |
| mp65 E39I | FG9 EF1α Puro | HA | M. Milanovic, Giessen |
| mp65 E39I/R302E | FG9 EF1α Puro | HA | M. Milanovic, Giessen |
| pRSV Rev | pUC118 | None | E. Burstein, Dallas |
| pMDLg/pRRE | pMD | None | E. Burstein, Dallas |
| pHCMV | pHCMV-VSV-G | None | E. Burstein, Dallas |

### 2.1.4.3 Plasmids for luciferase reporter gene assays

| cDNA | Vector | Supplier |
|---|---|---|
| (κB)x3-luc | pGL3basic | M.L.Schmitz, Giessen |
| (Gal4)x5-luc | pGL3basic | M.L Schmitz, Giessen |

### 2.1.5 Oligonucleotides

| Oligos for point mutagenesis of p65 | Sequence (5’ → 3’; mutated codon in bold) |
|---|---|
| p65 S276A_for. | TGCGGCGGCCT**GCC**GACCGGGAGCTCAG |
| p65 S276A_rev. | TCCCGGTC**GGC**AGGCCGCCGCAGCTGCA |
| p65 S276E_for. | CAGCTGCGGCGGCCT**GAG**GACCGGGAGCTCAGTG |
| p65 S276E_rev. | GAGCTCCCGGTC**CTC**AGGCCGCCGCAGCTGCATG |
| p65_S468A_for. | TTCACAGACCTGGCA**GCC**GTCGAC |
| p65_S468A_rev. | CTCGGAGTTGTCGAC**GGC**TGCCAGG |

| | |
|---|---|
| m_p65E39I_for. | CATGCGATTCCGCTATAAATGC**ATA**GGGCGCTCAGCGGGCAG |
| m_p65E39I_rev. | GAATACTGCCCGCTGAGCGCCC**TAT**GCATTTATAGCGGAATCGC |
| m_p65R302E _for. | GCCACCGGATTGAAGAGAAG**GAG**AAAAGGACCTATGAGACCTTC |
| m_p65R302E_rev. | CTTGAAGGTCTCATAGGTCCTTTT**CTC**CTTCTCTTCAATCCGGTG |

| **Oligo for EMSA** | **Sequence (5' → 3')** |
|---|---|
| κB | AGTTGAGGGGACTTTCCCAGGC (NF-κB site underlined) |

| **Oligos for qRT-PCR Gene target (mouse)** | **Sequence (5' → 3')** |
|---|---|
| *β-Actin*_for. | GAGATTACTGCTCTGGCTCCTA |
| *β-Actin*_rev. | TCATCGTACTCCTGCTTGCT |
| *Ccl9*_for. | CTGGGTCTGCCCACTAAGAAG |
| *Ccl9*_rev. | AATTTCAAGCCCTTGCTGTGC |
| *Ptgs2*_for. | TCTCCAACCTCTCCTACTAC |
| *Ptgs2*_rev. | GCACGTAGTCTTCGATCACT |
| *Cxcl10*_for. | AATCATCCCTGCGAGCCTAT |
| *Cxcl10*_rev. | TTTGGCTAAACGCTTTCATT |
| *Nfκbiα*_for. | CGCAGACCTGCACACCCCAG |
| *Nfκbiα*_rev. | GGAGGGCTGTCCGGCCATTG |
| *Il-6*_for. | TGGATGCTACCAAACTGGAT |
| *Il-6*_rev. | GGACTCTGGCTTTGTCTTTC |
| *Junb*_for. | TCACGACGACTCTTACGCAG |
| *Junb*_rev. | GGACCCTTGAGACCCCGATA |
| *Mip2*_for. | AGTGAACTGCGCTGTCAATG |
| *Mip2*_rev. | CTTCAGGGTCAAGGCAAACT |
| *Mmp10*_for. | CAGGAATTGAGCCACAAGTTGA |
| *Mmp10*_rev. | AGCCAGCTGTTGCTCTTCAGTA |
| *Saa3*_for. | CTGTTCAGAAGTTCACGGGAC |
| *Saa3*_rev. | AGCAGGTCGGAAGTGGTT |
| *Tlr-2*_for. | GGAGCATCCGAATTGCATCAC |

| | |
|---|---|
| *Tlr-2*_rev. | GCGTTTGCTGAAGAGGACTG |
| *Tnfaip3*_for. | GGTGACCCTGAAGGACAGTG |
| *Tnfaip3*_rev. | TCAAACCTACCCCGGTCTCT |

### 2.1.6 Antibiotics

| Name | Final conc. | Supplier |
|---|---|---|
| Ampicillin | 100 µg/ml | Sigma |
| Kanamycin | 50 µg/ml | Sigma |
| Puromycin | 2 µg/ml | Invivogen |
| Penicillin/Streptomycin | 10000 U/ml | Cell concepts |

### 2.1.7 Enzymes

| Enzyme | Supplier |
|---|---|
| Calf intestine alkaline phosphatase (CIAP) | Fermentas |
| DNase I (RNase-free) | Fermentas |
| Proteinase K | Sigma |
| RNase A | Sigma |
| Restriction enzymes | Fermentas and New England Biolabs |
| RiboLock RNase inhibitor | Fermentas |
| Shrimp alkaline phosphatase (SAP) | Roche |
| SuperScript$^{TM}$II reverse transcriptase | Invitrogen |
| T4 DNA ligase | Fermentas |
| T4 DNA polymerase | Fermentas |
| T4 polynucleotide kinase (T4 PNK) | Fermentas |
| *Taq* DNA polymerase | Fermentas |
| Trypsin | PAA Laboratories |
| Thermolysin | Promega |

### 2.1.8 Inhibitors

| Name | Final conc. | Target | Supplier |
|---|---|---|---|
| Aprotinin | 10 µl/ml | Serine proteases | Sigma |
| Calyculin A | 50 nM | Phosphatases | Sigma |
| Cycloheximide (CHX) | 10 ng/µl | 60S ribosomal unit | Sigma |
| GA | 1µM | Hsp90 | Invivogen |

| | | | |
|---|---|---|---|
| Leupeptin | 10 µl/ml | Proteases | Sigma |
| MG132 | 5 µM | Proteasome | Sigma |
| PiB | 2 µM | Pin1 | Sigma |
| VER | 40 µM | Hsp70 | Santa Cruz Biotech |

### 2.1.9 Kits

| Kit | Supplier |
|---|---|
| Absolute$^{TM}$ QPCR SYBR Green Mixes | Thermo |
| JETquick gel extraction spin kit | Genomed |
| JETstar plasmid purification kit (midi/maxi) | Genomed |
| LongRange PCR kit | Qiagen |
| QuikChange site-directed mutagenesis kit | Stratagene |
| RNase Mini kit | Qiagene |

### 2.1.10 Chemicals

| Substance | Supplier |
|---|---|
| Acetic acid | Roth |
| Acetone | Roth |
| Acrylamide/Bisacrylamide | Roth |
| Ammonium persulfate (APS) | Bio-Rad |
| Bromophenol blue | Merck |
| Boric acid | Fluca |
| Calcium chloride ($CaCl_2$) | Roth |
| Coomassie (Bradford) Protein Assay | Thermo Scientific |
| Dimethyl sulfoxide (DMSO) | Fluka |
| Di-*tert*-butyl peroxide (DTBP) | Sigma |
| 1,4-Dithiothreitol (DTT) | Acros Organics |
| Ethanol | Roth |
| Ethidium bromide | Roth |
| Ethylenediaminetetraacetic acid (EDTA) | Roth |
| Ethylene glycol tetraacetic acid (EGTA) | Fluka |
| Ficoll 400 | Serva |
| Glycerol | Roth |
| β-Glycerophosphate | Acros Organic |

| | |
|---|---|
| Glycine | Roth |
| Guanidine hydrochloride (Gu-HCl) | Roth |
| HEPES | Sigma |
| Hexadimethrine bromide (Polybrene) | Sigma |
| Hoechst 33342 | Invitrogen |
| Hydrochloride acide 37% (HCl) | Roth |
| IPTG | Roth |
| Imidazole | Fluka |
| Isopropanol | Merck |
| Kaiser's glycerol gelatine | Merck |
| Magnesium carbonate ($MgCO_3$) | Sigma |
| Magnesium chloride ($MgCl_2$) | Merck |
| Magnesium sulfate ($MgSO_4$) | Fluka |
| β-Mercaptoethanol | Fluka |
| Methanol | Roth |
| Nonidet P40 (NP-40) | Roche |
| Paraphormadehyde | Roth |
| Phenylmethanesulfonyl fluoride (PMSF) | Fluka |
| Polyethylenimine (PEI) | Sigma |
| Potassium acetate | Roth |
| Potassium chloride (KCl) | Roth |
| Potassium dihydrogene phosphate ($KH_2PO_4$) | Fluka |
| Potassium hydroxide (KOH) | Merck |
| Sodium azide ($NaN_3$) | Roth |
| Sodium chloride (NaCl) | Roth |
| Sodium dihydrogen phosphate ($NaH_2PO_4$) | Merck |
| Sodium dodecyl sulfate (SDS) | Bio-Rad |
| Sodium fluoride (NaF) | Roth |
| Sodium hydroxide (NaOH) | Merck |
| Sodium orthovanadate ($Na_3VO_4$) | Sigma |
| *N,N,N',N'*-Tetramethylethylendiamine (TEMED) | Bio-Rad |
| Tris base | Acros Organics |
| Triton X-100 | Sigma |

| | |
|---|---|
| Tryptone | AppliChem |
| Tween 20 | Gerbu |
| Urea | Roth |

### 2.1.11 Other reagents

| Substance | Source |
|---|---|
| Agar | AppliChem |
| Agarose | AppliChem |
| [$\gamma$-$^{32}$P]ATP | PerkinElmer |
| Bovine serum albumin (BSA) | Sigma |
| DMEM high glucose medium | Cell concepts |
| Fetal calf serum (FCS) | Cell concepts |
| GeneRuler 100bp DNA ladder | Fermentas |
| GeneRuler 1 kb DNA ladder | Fermentas |
| L-Glutamine | Cell concepts |
| Goat serum | Sigma |
| Lipofectamine 2000 | Invitrogen |
| Ni-NTA agarose | Qiagen |
| Oligo(dT)$_{12-18}$ primer | Invitrogen |
| OptiMEM | Gibco |
| PageRuler Plus prestained protein ladder | Fermentas |
| Protein G Plus/Protein A- Aagarose | Calbiochem |
| Recombinant IL-1 | Dr. Michael Kracht, Giessen |
| Recombinant TNF | Dr. H. Wajant, Würzburg |
| Rotifect | Roth |
| Skim milk powder | Merck |
| Trypsin/EDTA | PAA Laboratories |
| Western Lightning ECL solutions | Perkin Elmer |
| Yeast extract | AppliChem |

### 2.1.12 Buffers and other solutions

All buffers and solutions were prepared with deionized MilliQ water. For molecular biological methods, MilliQ water was autoclaved. Recipes for buffers and solutions are described within the corresponding section describing the method.

## 2.2 Methods in molecular biology

### 2.2.1 Transformation of chemically competent *E.coli*

Various chemically competent strains of *E.coli* were transformed taking an aliquot of bacterial cells, previously stored at -80˚C. The aliquot was thawed on ice and 50 µl of bacterial cells were mixed with up to 1 µg of plasmid DNA. The suspension was incubated for 20 min on ice. The uptake of DNA was allowed by heat shock at 42˚C for 90 seconds (sec), followed by incubation on ice for 2 min. The bacteria were cultured in 500 µl of Luria Bertani (LB) medium without antibiotics for 60 min at 37˚C on the shaker. Bacterial cells were centrifuged (3 min, 3500 rpm at 20ºC) and 300 µl of LB medium was discarded. The cell pellet was resuspended in remaining LB medium and plated on LB agar plates supplemented with antibiotic. Colonies were grown overnight at 37˚C.

**Luria Bertani (LB) medium:** 1% (w/v) tryptone; 0.5% (w/v) yeast extract; 1% (w/v) NaCl

**LB agar plates:** LB medium supplemented with 1.5% (w/v) agar and antibiotics

### 2.2.2 Storage of *E.coli*

Transformed bacterial cells of an overnight culture were mixed carefully with the same volume of 96% (v/v) glycerol by pipetting. The created bacterial stocks were stored at -80˚C and can be used for the inoculation of new overnight cultures.

### 2.2.3 Preparation of chemically competent *E.coli*

Competent *E.coli* cells were prepared using the $CaCl_2$ method. Antibiotic-free LB medium (10 ml) was inoculated with Top10 *E.coli* (taken from the glycerol stock) and the culture was shaken overnight at 37˚C. The overnight culture (10 ml) was the morning after diluted with 90 ml of pre-warmed antibiotic-free LB medium and grown at 37˚C with shaking until an optical density ($OD_{600\ nm}$) of 0.5-0.7 was reached. All subsequent steps were performed at 4˚C on ice. After the 15 min incubation period, cells were collected by centrifugation (15 min, 4000 rpm at 4ºC). The medium was discarded and the pellet was carefully resuspended in 50 ml of a sterile and ice-cold 0.1 M $CaCl_2$ solution, avoiding foaming. Cells were incubated on ice for 30 min and again pelleted by centrifugation (15 min, 4000 rpm at 4ºC). The supernatant was discarded and the cells were gently

resuspended in 3 ml of sterile 0.1 M $CaCl_2$ containing 10% glycerol (v/v). Competent *E.coli* cells were aliquoted (100µl) into the sterile 1.5 ml tubes, shock frozen in liquid nitrogen and stored at -80˚C until use.

### 2.2.4 Isolation of plasmid DNA from transformed *E.coli*

For the isolation of plasmid DNA a method based on alkaline lysis was used. Bacterial *E.coli* cells containing the plasmid of interest were first cultured overnight in LB medium containing the appropriate antibiotic. For Mini-preps, 2 ml of overnight culture was centrifuged, the supernatant was discarded and the pellet was suspended in 200 µl of EDTA-containing physiological buffer P1, supplemented with RNase A. The following step included the lysis of bacterial cells by adding the 200 µl of lysis buffer P2. During the 5 min incubation at room temperature (20ºC), SDS contained in the P2 buffer dissolves the lipid components from the cell membrane, as well as cellular proteins. The high alkaline conditions denature chromosomal and plasmid DNA. To neutralize the solution, 200 µl of pre-chilled buffer P3 was added and the tubes were mixed by inversion. Potassium acetate returns the pH to neutral allowing the renaturation of DNA strands. Large chromosomal strands partially renature, while the single-stranded plasmid DNA completely renaturates into dsDNA molecules and cellular proteins precipitate. Lysates were incubated for 20 min on ice and cleared from debris by centrifugation (10 min, 13000 rpm at 4ºC). The supernatant was transferred into a new tube and iso-propanol was added (final concentration 70%). The DNA was pelleted by centrifugation (10 min, 13000 rpm at 4ºC) and washed with 300 µl 70% (v/v) ethanol which removed some remaining salts and SDS from the preparation. After the centrifugation step (10 min, 13000 rpm at 4ºC), pelleted DNA was air-dried at 20ºC for 10 min. Plasmid DNA was disolved in 30 µl sterile water and stored at -20˚C.

Medium and large scale plasmid DNA purifications were performed using the JetStar 2.0 Plasmid Purification Kit (Genomed) according to manufactures instructions. The purification is also based on alkaline lysis. Lysates were centrifuged and supernatants were passed through JetStar 2.0 anion-exchange columns. The negatively charged phosphates on the DNA-backbone interact with the positive charges on the resin surface. Once the lysate ran through the column by gravity flow, the column was washed with washing buffer allowing the removal of RNA, proteins, carbohydrates and other impurities, while the

plasmid DNA remains bound to the resin. The plasmid DNA was eluted under high salt conditions and precipitated from the eluate with isopropanol and 70% ethanol.

**Buffer P1:** 50 mM Tris/HCl (pH 8.0); 10 mM EDTA; 100 µg/ml RNase A

**Buffer P2:** 200 mM NaOH; 1% (w/v) SDS

**Buffer P3:** 3 M potassium acetate (pH 5.5)

The concentration of a DNA purified by columns was quantified in a spectrophotometer by measuring the absorbance of the sample at a wavelength of 260 nm. The ratio of the absorbance at 260 and 280 nm ($OD_{260/280}$) was used to assess DNA purity. An $OD_{260/280}$ ratio between 1.7 and 2.0 represented a high-quality sample.

### 2.2.5 Polymerase Chain Reaction (PCR)

#### 2.2.5.1 Amplification of DNA fragments for cloning

PCR was used for amplification of desired DNA fragments. The method is based on the usage of two oligonucleotide primers that hybridize to complementary target regions on opposite DNA strands and flank the target sequence. Oligonucleotide primers were designed with additional non-annealing DNA sequence containing specific endonuclease restriction sites at the 5' ends of each primer, allowing the direct cloning of DNA fragment. For efficient amplification of DNA fragments, the QIAGEN LongRange PCR Kit was used according to manufacturers suggestions. PCR reaction mix consistent of:

| **Reagent** | |
|---|---|
| Template DNA | 20 – 100 ng |
| LongRange PCR buffer (10x) | 5 µl |
| dNTP mix | 500 µM |
| forward primer | 0.4 µM |
| reverse primer | 0.4 µM |
| LongRange PCR enzyme mix | 2 units |
| MilliQ water | up to 50 µl |

PCR reaction was performed in thermal cycler using the following program:

| | | |
|---|---|---|
| Initial denaturation | 93˚C, 3 min | |
| Denaturation | 93˚C, 20 sec | |
| Primer annealing | 55 - 60˚C; ~ 5˚C below Tm of primers, 30 sec | 35 cycles |
| Elongation | 68˚C, 1 min/kb | |
| Final elongation | 68˚C, 5 min | |
| Storage | 4˚C until use or -20˚C for long term storage | |

After amplification, the PCR product was analysed by agarose gel electrophoresis, purified from the agarose gel (2.2.1.7) and digested with restriction enzymes (2.2.1.8).

### 2.2.5.2 Site-directed point mutagenesis

In order to examine the importance of single amino acids and their PTMs for protein functions, site-directed point mutants were produced. Single amino acids were changed using the QuikChange site-directed mutagenesis kit. Specific oligonucleotides, carrying the changed sequence, were previously designed. PCR reaction mix for point-mutagenesis contained:

| **Reagent** | |
|---|---|
| Template DNA | 20-100 ng |
| 10x reaction buffer | 5 µl |
| Quik solution | 3 µl |
| 10 mM dNTP mix | 1 µl |
| forward primer | 125 ng |
| reverse primer | 125 ng |
| *Pfu ultra* DNA polymerase | 2.5 units |
| MilliQ water | up to 50 µl |

Mutagenesis PCR was performed using the following program:

| | | |
|---|---|---|
| Initial denaturation | 95˚C, 2 min | |
| Denaturation | 95˚C, 30 sec | 18 cycles |
| Primer annealing | 55˚C, 1 min | |
| Elongation | 68°C, 1 min/kb | |
| Final elongation step | 68ºC, 7 min | |

Non-mutated template DNA from the PCR reaction was removed by adding 1 µl of *Dpn*I (10 U/µl) to the reaction mix, followed by incubation for at least 2 h at 37˚C. To increase the purity of mutated plasmid DNA, the DNA was separated on an agarose gel and purified (see 2.2.1.7). The amplified plasmid DNA was transformed into Top10 *E.coli* cells (see 2.2.1.1) and cells were plated on LB agar containing the appropriate antibiotic. Plasmid DNA was purified (see 2.2.4.1) and the presence of desired point mutation(s) was confirmed by DNA sequencing or by restriction digestion, in case that diagnostic restriction sites were included in primers sequences.

### 2.2.6 Agarose gel electrophoresis

Agarose gel electrophoresis was performed for the analysis of digested vectors and PCR products. Agarose gels (0.8 - 2.0% (w/v)) were prepared dissolving the powdered agarose in a TRIS-acetate-EDTA (TAE) buffer. The agarose solution was heated in a microwave until it was completely melted, left for about 5 min to cool down and ethidium bromide was added (final concentration 0.2 µg/ml) to the agarose solution. This fluorescent dye intercalates between bases of nucleic acids and allows the detection of a DNA under the UV light. DNA samples were mixed with 6x DNA sample buffer before gel loading. Gels were run in TAE buffer at a constant voltage of 80 V or maximally 70 V for preparative agarose gels. Separation of DNA fragments was analysed on an UV-transilluminator.

**50x TAE buffer:** 2M Tris (pH 8.3); 1 M acetic acid; 50 mM EDTA

**6x DNA sample buffer:** 60 mM EDTA (pH 8.0); 60% (w/v) glycerol; 0.09% (w/v) bromphenol blue

#### 2.2.6.1 DNA extraction from agarose gel

The DNA fragment of interest was located at low UV radiation (reduced intensity), excised from the agarose gel with a razor blade and the DNA was extracted using the JETquick gel extraction kit (Genomed) according to manufacturers instructions. Briefly, the gel slice was solubilised by adding solution L1 and heated to 50°C for 15 min. The mixture was loaded into a JETquick spin column where the DNA bound to the resin. After several washing steps with the solution L2, DNA fragments were eluted from the resin with 30 µl of sterile water. The yield and purity of the extracted DNA was tested by agarose gel electrophoresis.

### 2.2.7 Digestion of DNA with restriction enzymes

For the insertion of amplified DNA fragment into the vector, both DNA plasmid and PCR products were digested with restriction enzymes to generate complementary end with single-stranded overhangs. In most cases the digestion was performed with two restriction enzymes in the recommended restriction buffer. A reaction mix for the analytical DNA digestion included:

| **Reagent** | |
|---|---|
| DNA (1 µg/µl) | 2 µl |
| 10x restriction buffer | 1 µl |
| Restriction enzyme 1 (10 U/µl) | 5 units |
| Restriction enzyme 2 (10 U/µl) | 5 units |
| MilliQ water | up to 10 µl |

After the incubation for at least 2 h at the optimal temperature for restriction enzyme(s), products were analysed by agarose gel electrophoresis (see 2.2.1.7) and purified for cloning when necessary (see 2.2.1.8). If a single restriction enzyme was used, 5'-phosphate ends of linearised DNA plasmid were dephosphorylated using CIAP in order to avoid re-ligation of the plasmid DNA. A reaction mix for the DNA dephosphorylation included:

| **Reagent** | |
|---|---|
| Linearized plasmid DNA (1 µg/µl) | 2 µl |
| 10x CIAP reaction buffer | 1 µl |
| CIAP (1 U/µl) | 1 unit |
| MilliQ water | up to 10 µl |

After the incubation on 37˚C for 30 min, reaction was stopped by heating for 10 min at 85°C.

### 2.2.8 Ligation of DNA fragments

To assure the best outcome, ligation mixtures were prepared with a molar ratio of vector to insert of 1:3 and 1:5. The amount of DNA insert was calculated by converting the molar ratios to mass ratios using the formula:

*Amount of the DNA insert= ((ng of vector x kb size insert) / kb size of vector) x molar ratio insert/vector*

The ligation mix contained:

| **Reagent** | |
|---|---|
| Cutted vector | X |
| DNA insert | X |
| 10x T4 DNA ligase buffer | 2 µl |
| T4 DNA ligase | 1 µl |
| MilliQ water | up to 20 µl |

After the incubation period of 2 h at 20ºC or overnight at 16˚C, the ligation reaction mixtures were used directly for transformation of chemically competent *E.coli* cells.

### 2.2.9 RNA extraction

The RNeasy kit was used for the isolation of total RNA from cells according to manufacturers instructions. Cells grown on a 10 cm cell-culture dish were harvested, washed twice in PBS and lysed with 600 ml of RLT buffer. Lysates were homogenized by passing the lysate through a needle for several times. The same volume of 70% ethanol (600 ml) was added to the lysate and the mixture was loaded on RNeasy spin columns placed in 2 ml collection tubes. After the centrifugation (15 sec, > 10000 rpm at 20ºC), the flow-through was discarded and the RNA bound to columns was washed once with RW1 and twice with RPE buffer. Each washing step was followed by centrifugation (15 sec, > 10000 rpm at 20ºC, and last centrifugation step: 2 min, 13000 rpm at 20ºC). The total RNA was eluted by adding 30 µl of RNase-free water in the center of silica membrane. Columns were placed into the fresh collection 1.5 ml tubes and centrifuged with 13000 rpm for 1 min at 20ºC. The yield and purity of isolated RNA was quantified in a spectrophotometer by measuring the absorbance of the sample at a wavelength of 260 nm. The ratio of the absorbance at 260 and 280 nm ($OD_{260/280}$) was used to assess DNA purity. An $OD_{260/280}$ ratio around 2.0 represented a high-quality sample; RNA was stored at -80˚C.

### 2.2.10 Complementary DNA (cDNA) synthesis

Reverse transcription of RNA into cDNA was done using the reagents and the protocol displayed in the Table 2.1 below.

**Table 2.1** Reverse transcription reaction

| **Reagent** | |
|---|---|
| RNA | 1 µg |
| dNTP mix (10 mM each) | 1 µl |
| oligo$(dT)_{12-18}$ primer (stock concentration 0.5 µg/µl) | 1 µl |
| MilliQ water | up to 12 µl |

After heating to 65˚C for 5 min, the following reagents were added :

| **Reagent** | |
|---|---|
| 5x First-strand buffer | 4 µl |
| 0.1 M DTT | 2 µl |
| RiboLock RNase inhibitor | 40 units |
| SuperScript II reverse transcriptase | 200 units |

The samples were incubated in the thermocycler at 42ºC for 55 min and thereafter the reaction was inactivated by heating at 70ºC for 15 min. The cDNA was diluted with sterile water to a volume of 100 µl and used as a template for quantitative real-time PCR.

### 2.2.11 Quantitative real-time PCR (qRT-PCR)

Relative quantification of mRNA levels was measured by qRT-PCR. Target-gene specific oligonucleotides were designed that were flanking introns, thus avoiding the detection of genomic DNA. PCR products were detected with SYBR green, a commonly used fluorescent DNA binding dye which binds to all newly synthesized dsDNA complexes. The fluorescence is measured at the end of each PCR cycle so the intensity of the fluorescence reflects the amount of dsDNA present in the sample, allowing quantification of specific nucleic acid sequences. The qRT-PCR reaction was performed using an ABI 3000 RT-PCR cycler (Applied Biosystems), in 96 well plates.

qRT-PCR reaction mix included:

| **Reagent** | |
|---|---|
| Template cDNA | 20 ng |
| 1x SYBR green ROX mix | 5µl |
| forward primer | 70 nM |
| reverse primer | 70 nM |
| MilliQ water | up to 10 µl |

The qRT-PCR reaction was performed using the following program:

| | | |
|---|---|---|
| Enzyme activation | 95˚C, 15 min | |
| Denaturation | 95˚C, 15 sec | 40 cycles |
| Primer annealing | 60˚C, 30 sec | |
| Elongation | 72˚C, 1 min | |

To confirm the specificity of reaction, a melting curve analysis was performed under the following program:

| | | |
|---|---|---|
| Denaturation | 95˚C, 30 sec | |
| Starting temperature | 60˚C, 30 sec | |
| Melting step | 60˚C, 10 sec | 80 cycles |

All experiments were performed in triplicates and quantification was done using the comparative delta delta threshold cycle ($\Delta\Delta C_{t)}$ method. Data were normalized to the housekeeping gene β-Actin and the resulting $\Delta C_t$ values were compared to a sample that was chosen as a calibrator (e.g. wild type cells). The relative expression level was calculated using the formula:

$$Fold\ change = 2^{-\Delta Ct\ sample\ A} / 2^{-\Delta Ct\ calibrator}$$

## 2.3 Methods in cell biology

### 2.3.1 Cultivation of eukaryotic cell lines

Cell lines used in this study were grown in complete Dulbecco's modified medium (DMEM) (see 2.1.8.2) and incubated at 37˚C in an atmosphere of 5% $CO_2$. Cells were

cultured in 75 or 175 $cm^2$γ-irrediated cell-culture flasks for 2-3 days to reach a confluency of 70-80%. The adherent cells that stick to the surface were washed once with pre-warmed PBS and detached from the flask surface by adding Trypsin/EDTA, followed by incubation for 2-3 min at 37˚C. Trypsin activity was stopped by adding complete DMEM. Cells were seeded into a new flask in an appropriate dilution. Easily detached adherent cell lines such as HEK-293 and HEK-293T cells were detached by intensive pipetting and seeded into new flask. If necessary, the cells were pelleted by centrifugation (1300 rpm, 3min at 20˚C), resuspended in the desired volume of complete DMEM and seeded.

**PBS:** 137mM NaCl; 1.5 mM $KH_2PO_4$; 2.7mM KCl; 8.1 mM $Na_2HPO_4$ (pH 7.4)

**DMEM complete:** DMEM high glucose (4.5 g/L); 2 mM L-glutamine;
1% (v/v) penicillin/streptomycin;
10% (v/v) heat inactivated (56˚C, 30 min) FCS

### 2.3.2 Freezing and thawing of cells

For long-term storage, exponentially grown cells were collected by centrifugation (1300 rpm, 3 min at 20˚C), washed once in PBS, collected again by centrifugation and resuspended in 1ml of freezing medium. Cells were aliquoted in labeled cryo tubes, transferred to a cooling device ensuring slow cooling and transferred to -80˚C for one day. The next day, cells were transferred for long-term storage at -150˚C.

Frozen cryo tubes were thawed at 37˚C in the water bath. Cells were washed in 10 ml of pre-warmed complete DMEM, followed by centrifugation (1300rpm, 3 min at 20˚C). After removal of the supernatant the pellet containing the collected cells were resuspended in 10 ml of complete DMEM and seeded into a 75 $cm^2$ cell-culture flask.

**Freezing medium:** 90% (v/v) FCS; 10% (v/v) DMSO

### 2.3.3 Transfection of mammalian cells

Introduction of plasmid DNA into eukaryotic cells was performed using polyethylenimine (PEI). As a polycationic transfection reagent, it binds to DNA, neutralizes its negative charge, condenses its structure and forms a PEI-DNA complex that is easily introduced into the cell by endocytosis.

One day prior to transfection, cells were seeded in 10 cm dishes in order to reach 50-60% confluency at the day of transfection. For the transfection, the ratio of DNA and transfection reagent was always 2 µl of PEI per 1 µg of DNA (PEI stock concentration1 µg/µl). Total DNA amounts were kept equal in all transfections by adding empty vector. All the steps were performed under the hood.DNA was mixed with serum- and antibiotic free DMEM (50 µl per 1 µg of DNA) and incubated for 5 min at 20ºC. In the other 1.5 ml tube, PEI was diluted with the serum- and antibiotic free DMEM and incubated for 5 min at 20ºC. After 5 min, diluted PEI was added to DNA mixture and incubated for 20 min at 20ºC allowing formation of PEI-DNA complex. In the meantime, the complete medium from the cultivated 10 cm dishes was aspirated off, cells were washed twice with pre-warmed PBS and 5 ml of the antibiotic free DMEM (transfection medium) was put over cultured cells. The PEI-DNA mixture was added in a drop-wise manner to cells. After 3-6 h, the transfection medium was aspirated off from plates and replaced by 6 ml of complete DMEM. After 36-48 h of incubation at 37˚C, cells were ready for the lysis.

**DMEM antibiotic-free:** DMEM high glucose (4.5 g/L); 2 mM L-glutamine; 10% (v/v) heat inactivated (56˚C, 30 min) FCS

Transfection mix for protein overexpression contained:

| **Reagent** | |
|---|---|
| plasmid DNA | 1-3 µg |
| PEI (stock concentration 1 µg/µl) | 2-9 µl |
| DMEM serum reduced | 50 µl per 1 µg of plasmid DNA |
| DMEM antibiotic-free | 2 or 5 ml, for 6 or 10 cm cell culture dish |

### 2.3.4 Production of lentiviruses and infection of cells

Production of lentiviral particles of the 3$^{rd}$ generations was done in HEK-293T packaging cells. The FG9EF-1a-Puro expression vector encoding p65 or its mutants were transfected together with packaging vectors pMDLg/pRRE, pRSV-Rev and pHCMVG, in a ratio of 1:1:1:1 (5µg of the each construct). Cells were seeded in 10 cm dishes to reach a confluence of 80% on the day of transfection. As a transfection reagent was used Lipofectamine2000according to the procedure suggested by the manufacturer.

Lipofectamine is a cationic lipid and facilitate transfection during the early steps of the process by mediating DNA condensation and DNA/cellular interactions. In one sterile tube five micrograms of each of four vectors (in total 20 µg of DNA) were mixed with 1.5 ml OptiMEM. In the other sterile tube 40 µl of Lipofectamine2000 (DNA – Lipofectamine2000 ratio of 1:2) was mixed with 1.5 ml OptiMEM. After the 5 min incubation at 20ºC, the diluted DNA and Lipofectamine2000 were combined and incubated for 20 min to allow formation of Lipofectamine-DNA complexes. Complete medium was removed from each 10 cm dish and replaced with antibiotic-free DMEM containing 2.5% (v/v) FCS and 3 ml of transfection mixture were added to the cells. After 4-6 h, the transfection medium was totally replaced by DMEM complete medium. Viruses were collected 48 h post-transfection, filtered through a 0.45 µm filter and mixed with polybrene (final concentration 5µg/ml) to increase the efficiency of infection. Viruses were added over cells. One day after infection, medium with viruses was removed and replaced with the complete DMEM medium containing 2 µg/ml of puromycin (final concentration) for cell selection. Cells were kept under the puromycin selection for at least one week.

**DMEM serum reduced**
**for the lentivirus production:** DMEM high glucose (4.5 g/L); 2 mM L-glutamine; 1.5% (v/v) heat inactivated (56˚C, 30 min) FCS

Transfection mixture for lentivirus production included (for 10 cm cell culture dish):

| **Reagent** | |
|---|---|
| lentiviral vector | 5 µg |
| packaging vectors: pMDLg/pRRE | 5 µg |
| pRSV-Rev | 5 µg |
| pHCMVG | 5 µg |
| Lipofectamine2000 (stock concentration 1 µg/µl) | 40 µl |
| OPTI-MEM serum reduced | 3 ml |
| DMEM serum reduced | 7 ml |

### 2.3.5 Lysate preparation

#### 2.3.5.1 Lysis under denaturing conditions

Cells were scraped off, washed twice in ice-cold PBS and collected by centrifugation for 2 min, 2000 rpm at 4ºC). Cells were lysed in 100-200 µl of 1x SDS sample buffer and the lysate was boiled for 2 min at 95˚C. The DNA was fragmented by shearing (2 x 20 sec) using a Branson 250 sonifier. Lysates were boiled again at 95˚C for 5 min and used for Western blotting or frozen at -80˚C.

**5x SDS sample buffer:** 312.5 mM Tris/HCl (pH 6.8); 50% (v/v) glycerol; 25% (v/v) β-mercaptoethanol; 10% (w/v) SDS; 0.01% (w/v) bromphenol blue

#### 2.3.5.2 Lysis under native conditions - whole cell lysate

Whole cell lysates were prepared by lysing cells in the lysis buffer containing the non-ionic detergent NP-40. Cells were harvested and washed twice in ice-cold PBS. Cell pellets were resuspended in an appropriate volume of the NP-40 lysis buffer (approximately 4 volumes of NP-40 lysis buffer for 1 volume of cell pellet) and incubated 20 min on ice. Lysates were cleared by centrifugation (15 min, 13200 rpm, 4˚C) and supernatants were transferred into fresh tubes. Lysates were used for limited proteolysis assay, immunoprecipitation (IP), GST pull-down, luciferase reporter assay or were directly mixed with 5x SDS sample buffer at a ratio of 1:4 (5x SDS sample buffer : lysate), boiled for 5 min at 95˚C and analysed by Western blotting.

**NP-40 lysis buffer:** 20 mM Tris/HCl (pH 7.5); 150 mM NaCl; 1% (v/v) NP-40;
*freshly added:* 0.5 mM $Na_3VO_4$*;* 10 mM NaF; 1 mM PMSF; 10 µg/ml aprotinin; 10 µg/ml leupeptin

#### 2.3.5.3 Lysis under native conditions - subcellular fractionation

For preparation of cytosolic and nuclear protein extracts, cells were harvested after washing in cold PBS and collected by centrifugation. The pellet was suspended in cold extraction buffer A. After incubation on ice for 10 min, NP-40 was added in a final concentration of 0.25% (v/v) and cells were vortexed gently for 5 sec. Samples were centrifuged (10 min, 4200 rpm at 4˚C) and the supernatants (corresponding to cytosolic extracts) were collected and transfered to a fresh tubes. Cell pellets were washed once in

buffer A and cleared by centrifugation (10 min, 4200 rpm at 4˚C). For the preparation of nuclear extracts, pellets were resuspended very gently in extraction buffer C. After incubation on ice for 20 min and centrifugation (10 min, 13200 rpm at 4˚C), supernatants corresponding to nuclear extracts were transferred to fresh tubes. Cytosolic and nuclear extracts were further used for limited proteolysis assay, IP, electrophoretic mobility shift assay (EMSA) or they were mixed with 5x SDS sample buffer, boiled at 95˚C for 5 min and analysed by Western blotting.

**Buffer A**: 10 mM HEPES/KOH (pH 7.9); 10 mM KCl; 0.1 mM EDTA; 0.1 mM EGTA; 1 mM β-mercaptoethanol

**Buffer C:** 20 mM HEPES/KOH (pH 7.9), 400 mM NaCl; 1 mM EDTA; 1 mM EGTA; 1 mM β-mecaptoethanol

**10% NP-40 solution:** 10% (v/v) NP-40

### 2.3.6 Immunofluorescence (IF) staining

Intracellular localization of the proteins was analysed by IF. Adherent MEFs were grown on cover slips in 12-well plates to reach the confluence of 70% on the day of experiment. All washing and incubation steps were done under gentle shaking on 20ºC. The medium was aspirated off and the cells were washed twice with PBS for 5 min. Cells were fixed by adding 500 µl of freshly made 4% (w/v) paraformaldehyde in PBS and incubated for 15 min at 20ºC. After removal of the paraformaldehyde solution, cells were washed twice for 5 min with PBS. For cell permeability, cells were incubated with PBS containing 0.25% (v/v) Triton X-100 for 10 min. After the 3 washing steps with PBS, cells were blocked for 60 min by shaking in PBS containing 10% (v/v) goat serum. The blocking solution was removed and replaced with the primary antibody diluted in PBS containing 1% (v/v) goat serum. Cells were incubated overnight at 4˚C. Cells were washed four times with PBS after which were incubated for 60 min with fluorescence dye-coupled secondary antibodies diluted in PBS containing 1% (v/v) goat serum. Of note, cells were protected from light. Cells were again washed four times with PBS, still keeping them protected from light and a nuclear DNA was stained by incubation with Hoechst (1:1000 dilution in PBS) for 10 min. Cells were washed two times with PBS. Cover slips were mounted with one drop of pre-warmed Kaiser's glycerol gelatin on a surface of a microscope slide and gently pressed.

Slides were left at 4˚C overnight. The next day, cover slips and slides were cleaned and sealed with the nail polish. Microscope analysis was done using an inverted Nikon Eclipse 2000E microscope.

### 2.3.7 Luciferase reporter gene assays

To study transcriptional activity in cells, luciferase reporter assays were performed. Cells were transiently transfected with constructs in which a specific promoter sequence is fused to a luciferase gene. The luciferase gene encodes the enzyme luciferase, which catalyzes the oxidation of its substrate luciferin in a light producing reaction. Since the transcription of the luciferase gene is controlled by DNA elements allowing binding of a TF of interest, the amount of synthesized luciferase and hence the intensity of bioluminiscence represents the TF activity.

Cells were grown on 6 well plates and transfected with plasmids encoding the TF-reporter construct and if necessary, the corresponding TF. After 24-48 h post-transfection cells were stimulated with cytokines for 6 h or left untreated. Cells were washed with PBS and lysed in 100µl cold NP-40 buffer. Lysates were cleared by centrifugation (10 min, 13200 rpm at 4ºC), supernatants (10 µl) were mixed with luciferase buffer (10 µl) and bioluminiscence was immediately measured for 10 sec in a luminometer (Berthold Lumat LB 9507).

**Firefly luciferase assay buffer:** 0.47 mM *Photinius*-Luciferin; 530 µM ATP; 2.67 mM $MgSO_4$; 20 mM Tricine; 1.07mM $(MgCO_3)_4Mg(OH)_2 \times 5H_2O$; 0.1 mM EDTA; 270 µM Coenzyme A; 33.3 mM DTT

## 2.4 Biochemical methods

### 2.4.1 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE allows the separation of proteins in a gel matrix according to their molecular weight. Samples were mixed with 5x SDS sample buffer containing the anionic detergent SDS and β-mercaptoethanol in order to disrupt intra- and intermolecular protein interactions without breaking peptide bonds. The discontinuous SDS-PAGE employs two

gel phases. The upper stacking gel concentrates the proteins in a sharp band. A slightly acidic pH 6.8 in the stacking gel forms the ion gradient which results in migration of the SDS-coated proteins between $Cl^-$ (leading) and $Gly^-$ (trailing) ions, allowing their concentration in a sharp band. This region of the gel has larger pores and the gel matrix does not retard the protein migration during this step. Separation of proteins by size is achieved in a lower, resolving part of the gel. A shift to a higher pH 8.8 in the resolving gel accelerate migration of the trailing ions directly behind the leading ions, allowing proteins to separate by sieving effect due to the much smaller pore size of the resolving gel. Depending of the expected size of the protein of interest, resolving gels with different pore size were made.

**Table 2.2** Mixtures for preparation of different resolving and stacking SDS-polyacrylamide gels.

| | resolving | | | | | | stacking |
|---|---|---|---|---|---|---|---|
| **Solutions** | **6%** | **8%** | **10%** | **12%** | **13.5%** | **15%** | **4%** |
| 4x resolving buffer | 2.0 ml | 2.0 ml | 2.0 ml | 2.0 ml | 2.0 ml | 2.0 ml | - |
| 4x stacking buffer | - | - | - | - | - | - | 1 ml |
| 30% Acrylamide | 1.6 ml | 2.1 ml | 2.7 ml | 3.2 ml | 3.8 ml | 4 ml | 0.5 ml |
| $H_2O$ | 4.4 ml | 3.9 ml | 3.3 ml | 2.8 ml | 2.4 ml | 2 ml | 2.5 ml |
| 10% (w/v) APS | 50 µl | 50 µl | 50 µl | 50 µl | 50 µl | 50 µl | 50 µl |
| TEMED | 8 µl | 8 µl | 8 µl | 8 µl | 8 µl | 8 µl | 4 µl |

Samples were mixed with 5x SDS sample buffer at a ratio 4:1 (sample : buffer) and boiled for 5 min at 95˚C prior to loading on the gel. Electrophoresis was performed in 1x SDS running buffer. A current of 80 V was applied until proteins were concentrated in the stacking gel and once the separation started, it was increased up to 120 V. When resolving ubiquitinated proteins, a current of 80 V was kept during the entire process. For estimation of sample molecular weights, prestained protein markers were used. Separated proteins were visualized with Coomassie brilliant blue (see 2.2.3.2) or by Western blotting (see 2.2.3.3).

**10x SDS running buffer**: 250 mM Tris (pH 8.3); 2 mM Glycine, 1% (w/v) SDS

**4x resolving buffer**: 1.5 M Tris/HCl (pH 8.8); 0.4% (w/v) SDS

**4x stacking buffer**: 0.5 M Tris/HCl (pH 6.8); 0.4% (w/v) SDS

### 2.4.2 Coomassie brilliant blue staining of polyacrylamide gels

To visualize proteins on polyacrylamide gels, protein samples were electrophoretically separated, washed in 100 ml deionized water and stained by incubating the gel with Coomassie staining solution for 20 min under gentle agitation. To visualize proteins, the gel was rinsed in destaining solution several times.

**Coomassie staining solution**: 0.1% (w/v) Coomassie brilliant blue G-250; 40% (v/v) Methanol, 10% (v/v) Acetic acid

**Coomassie destaining solution**: 30% (v/v) Methanol; 20% (v/v) Acetic acid

### 2.4.3 Western blot and immune detection

Proteins separated by SDS-PAGE (see 2.2.3.1) were immobilized on polyvinylidene fluoride (PVDF) membranes by the Western blot method, which allows their detection by specific antibodies. For this aim, PVDF membrane was immersed in 100% methanol for a few sec until the entire membrane was translucent. The wet membrane was transferred to a vessel containing the transfer buffer and incubated for at least 2-3 min to allow membrane equilibration. Once equilibrated, the membrane was ready to bind proteins. In the meantime, Whatman papers were soaked in the transfer buffer. A blot "sandwich" consisting of two Whatman papers, one PVDF membrane, the gel and an additional Wathman paper placed on the top of the gel, was assembled on a semi-dry blotting apparatus (Bio-Rad). Air bubbles were carefully removed by pressing the blot "sandwich" very gently and the transfer was performed at the constant voltage of 24 V for 2 h. Afterwards, the membrane was incubated in blocking solution for 30 min at 20ºC, followed by incubation with appropriately diluted primary antibody for 2 h at 20ºC or overnight at 4˚C. Primary antibodies were diluted in 2% (w/v) skim milk powder or, in case of phospho-specific or acetyl-specific antibodies, in 1x Tris buffered saline (TBS-T) containing 3% (w/v) BSA. After washing the membrane 5 times in TBS-T buffer, the membrane was incubated with corresponding horseradish peroxidase (HRP)-coupled secondary antibodies for 2 h at 20ºC. All blocking, washing and antibody-incubation steps were performed under gentle shaking. Unbound secondary antibody was removed by washing the membrane five times in TBS-T and proteins were detected by autoradiography using an enhanced chemoluminiscence system.

**Transfer buffer:** 50 mM Tris/HCl, 40 mM Glycine, 20% (v/v) Methanol; 0.038% (w/v) SDS

**10x TBS-T:** 250 mM Tris (pH 7.4); 1.37 mM NaCl; 50 mM KCl;7 mM $CaCl_2$; 1 mM $MgCl_2$ x $2H_2O$; 0.1% (v/v) Tween 20

**2.4.4 IP**

IP was used to purify proteins or to study protein-protein interactions. Proteins of interest are precipitated by a specific antibody that is immobilized on a resin. Cells were harvested as described (see 2.2.2.5.1) and lysed under native conditions (see 2.2.2.5.2). Lysates were transferred to new tubes. An aliquot of the lysate (10%) was kept as an input control. The remaining lysate was pre-cleared adding 15 µl of A/G sepharose and incubated for 60 min at 4˚C on a rotating wheel. Samples were centrifuged (5 min, 2000 rpm at 4ºC) and cleared cell extracts were transferred to fresh tubes and supplemented with 1 µg precipitating antibody or control IgG. After incubation for 4 h at 4˚C on a rotation wheel, 30 µl of A/G sepharose were added to lysates. Samples were additionally incubated for 60 min (4˚C, rotation wheel) to allow binding of protein-antibody complexes to beads. Afterwards, beads were washed four times with 1 ml cold NP-40 lysis buffer by inverting tubes. After each washing step beads were collected by centrifugation (1 min, 2000 rpm at 4ºC). After the last washing step, 1 ml of a cold NP-40 lysis buffer was added over the beads and the slurry was transferred to a new eppendorf tube. The beads were collected by centrifugation, the supernatant was removed and precipitated proteins were eluted from beads by boiling in 1.5x SDS sample buffer for 5 min. Again, the samples were centrifuged (5 min, 13200 rpm at 4ºC) and the supernatants were transferred to fresh tubes and stored at -80˚C or directly analysed by Western blotting.

To detect weak protein-protein interactions, cells were treated the cross-linker DTBP. Cells were first washed once with cold PBS on the dish. A freshly prepared 0.5 mM DTBP solution in PBS was added to the cells and incubated for 30 min at 20ºC. After removing the DTBP solution cells were washed two times with PBS containing 200 mM TRIS/HCl (pH 7.5) for 10 min to stop the cross-linking reaction. Then, cells were harvested by scraping in PBS containing 200 mM TRIS/HCl and transferred into fresh tubes. After the two washing steps with PBS and collection by centrifugation (1 min, 2000 rpm at 4ºC) cells were lysed under native conditions by NP-40 buffer.

### 2.4.5 EMSA

EMSAs were used to study protein-DNA interactions. With this method it is possible to detect if a protein of interest is capable of binding to a specific DNA sequence. A radioactively labeled oligonucleotide is incubated with the protein of interest. As protein-free DNA oligonucleotides migrate faster on a native polyacrylamide gel, binding of the protein to the oligonucleotide will cause an upshift in the gel.

Cells were seeded on 10 cm cell culture plates and if necessary transfected with plasmids encoding wild type or mutated version of the protein of interest. After 24-48h post-transfection, cell lysates were prepared with TOTEX lysis buffer. Equal amounts of lysates were mixed with poly (dI-dC), BSA, $^{32}$P-labeled oligonucleotide (hot probe) and the 5x EMSA binding buffer in a final volume of 20 µl. The final NaCl concentration in the reaction mix was set to 100 mM. EMSA reaction mix contained:

| **Reagent** | |
|---|---|
| lysate | 10 µl |
| hot probe | 3 µl |
| poly (dI-dC) | 2 µg |
| BSA | 2 µg |
| 5x EMSA binding buffer | 4 µl |
| MilliQ water | up to 20 µl |

The mixture was incubated at 20ºC for 60 min. During that time the 4% (v/v) native polyacrylamide gel was prepared and pre-runned at 80 V for 30 min. After the incubation period, the reaction mixture was loaded onto a native 4% (v/v) polyacrylamide gel. Electrophoresis was performed in 0.5x Tris-borate-EDTA (TBE) buffer. First 30 min the gel was run at 100 V and afterwards the voltage was set to 150 V. The gel was run at 150 V for approximately 3 h. The apparatus was carefully disassembled and the gel was gently placed on Whatman paper, covered with transparent folia and dried for 2 h in Bio-Rad Gel Dryer. Without removing the folia, dry gel was exposed to an X-ray film over night on -80ºC.

Prior to EMSA experiment, oligonucleotides with NF-κB binding site (underlined) were labeled using polynucleotide kinase (PNK, Fermentas).

5’ –AGTTGAGGGGACTTTCCCAGGC- 3’
3’ –TCAACTCCCCTGAAAGGGTCCG- 5’

Reaction mix for radioactive labeling of oligos contained:

| **Reagent** | |
|---|---|
| Annealed oligos | 100 ng |
| [γ-$^{32}$ P] ATP (6000 Ci/mM, 10 µCi/µl) | 5 µl |
| 10x PNK Buffer A | 5 µl |
| PNK (10 U/µl) | 10 units |
| MilliQ water | up to 50 µl |

The mixture was incubated for 30 min at 37˚C. Excess [γ-$^{32}$ P] ATP was removed using a Sephadex G50 spin column equilibrated with Tris-EDTA (TE) buffer. The column was placed in a fresh collection tube. The reaction mix was added, centrifuged for 30 sec at 13200 rpm and flow through contained labeled product.

**TOTEX buffer:** 20 mM Hepes/KOH (pH 7.9); 350 mM NaCl; 20% (v/v) glycerol; 1% (v/v) NP-40; 1 mM $MgCl_2$; 0.5 mM EDTA; 0.1 mM EGTA;
*freshly added:* 0.5 mM $Na_3VO_4$; 10 mM NaF; 1 mM PMSF

**TE buffer:** 10 mM Tris-HCl (pH 8.0); 1 mM EDTA

**5x EMSA binding buffer:** 100 mM HEPES/KOH (pH 7.9); 300 mM NaCl; 1 mM DTT; 20% (v/v) Ficoll 400

**5x TBE buffer**: 445 mM Tris; 445 mM Boric acid; 10 mM EDTA

A mixture for a native 4% (v/v) polyacrylamide gel :

| **Solutions** | **Volume** |
|---|---|
| 5x TBE | 4.5 ml |
| 30% Acrylamide | 6 ml |
| $dH_2O$ | 34.5 ml |
| APS (10%) | 300 µl |
| TEMED | 37.5 µl |

### 2.4.6 Ni-NTA affinity purification

Binding of His residues on nickel nitrilotriacetic acid (Ni-NTA) allows the affinity purification of proteins tagged with 6 consecutive His residues (His-tag) under denaturing conditions. Ni-NTA purification was used to analyse protein ubiquitination. HEK-293T cells were transfected with expression vectors encoding the target protein together with a vector encoding the His-tagged Ub. Cells were harvested 48 h post-transfection and washed two times with cold PBS. To confirm protein expression, 20% of cells were directly lysed in 1x SDS sample buffer. The remaining cells were lysed in 800 µl NiNTA lysis buffer and the DNA was shared by sonication (2 x 20 sec). After centrifugation (10 min, 13200 rpm, 4˚C), supernatants were transferred to fresh tubes, mixed with 50 µl prewashed Ni-NTA beads and incubated for 2-4 h at 20ºC on a rotating wheel. Beads were first washed three times by resuspending beads in 1 ml Ni-NTA lysis buffer, followed by two washing steps in 1 ml Ni-NTA washing buffer**.** During washing steps, both buffers were supplemented with 10 mM β-mercaptoethanol and 25 mM imidazole. Between washing steps cells beads were collected by centrifugation (3 min, 2000 rpm at 20ºC). Bound proteins were eluted by boiling the beads in 50 µl elution buffer. The eluates were centrifuged (5 min, 13200 rpm at 4ºC), transferred into a fresh tube and analysed by Western blotting (see 2.2.3.3).

| | |
|---|---|
| **Ni-NTA lysis buffer:** | 50 mM Tris/HCl (pH 8.0); 8 M Urea; 300 mM NaCl; 50 mM $Na_2HPO_4$; 0.5% (v/v) NP-40 |
| **NiNTA washing buffer:** | 50 mM Tris/HCl (pH 8.0); 8 M Urea; 200 mM NaCl; 0.2% (w/v) SDS |
| **Elution buffer:** | 2.5x SDS sample buffer; 200 mM imidazole |

### 2.4.7 Limited proteolysis assay

Proteolysis is the breakdown of proteins into smaller polypeptides and amino acids by proteases. For limited proteolysis assays, the digestion reaction was performed under conditions which would enable just the partial digestions of proteins. This gives the possibility to indirectly measure possible changes in protein conformation. Trypsin and thermolysin were proteases used in these assays. The method is based on the property of protease to hydrolyse the accessible peptide bonds between specific amino acids. Trypsin

specifically cleaves the peptide bond at the C-terminus of the lysine or arginine. Thermolysin preferentially cleaves at the N-terminus of the hydrophobic residues leucine, phenylalanine, valine, isoleucine, alanine and methionine.

Cells were harvested and washed twice in ice-cold PBS. Cell lysates were prepared in NP-40 buffer or by fractionation into cytosolic and nuclear extracts. All buffers lacked protease inhibitors and limited proteolysis was conducted upon addition of trypsin and thermolysin. Depending on the cell type, different enzyme concentrations were needed (Table 2.3).

**Table 2.3** Conditions for limited proteolysis

| **Cell type** | **Trypsin (final concentration)** | **Thermolysin (final concentration)** |
|---|---|---|
| 293 T | 10 mM | 10 ng/µl |
| 293 | 10 mM | 10 ng/µl |
| MEF | 5 mM | 7 ng/µl |
| HeLa | 2.5 mM | 5 ng/µl |
| **incubation conditions** | 37˚C, 30 min | 37˚C, 20 min |

Proteolysis was terminated upon addition of 5x SDS sample buffer and boiling for 5 min at 95ºC. The digested material was separated by SDS-PAGE and analysed by immunoblotting.

# 3. Results

## 3.1 The cytokine-induced conformational change of the NF-κB p65 subunit is mediated by phosphorylation

### 3.1.1 Phosphorylation regulates the structural flexibility of NF-κB p65

The NF-κB subunit p65 belongs to the group of proteins with intrinsically disordered regions. The C-terminal half of p65, spanning the NLS and the TAD, is unstructured under physiological conditions [32, 180]. The degree of disorder in the entire p65 protein was analysed using the SPINE-D (Sequence based Prediction with Integrated Neural network for Disordered residues) program [261]. As revealed by the SPINE-D score plot, the region corresponding to DNA-binding loop L1 (residues 30-50) and the entire C-terminal half of the p65 are predicted to be intrinsically disordered (Fig. 3.1).

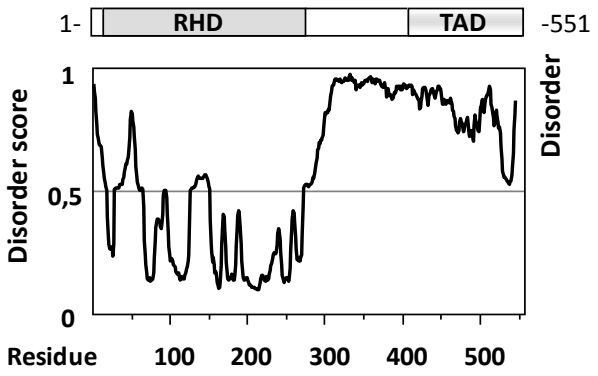


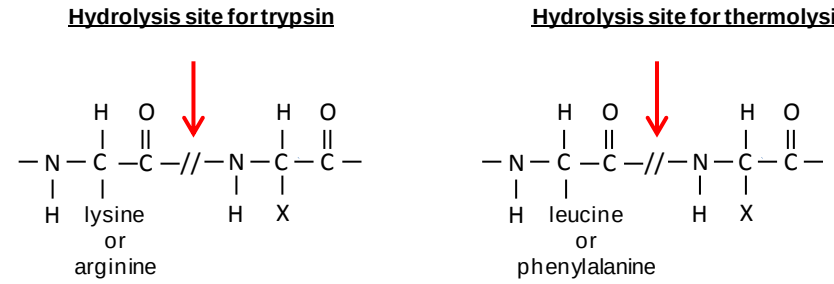

**Figure 3.1 Analysis of the disorder properties of p65.** The amino acid sequence of human p65 (NP_068810.3) was analysed using the trained neural network program SPINE-D for the occurrence of ordered and disordered regions. Values above the value of 0.5 are indicative for a disordered structure. Positions of amino acids (lower) and the schematic structure of p65 (upper) are indicated.

The intrinsic flexibility of disordered regions allows proteins to adopt alternative conformations enabling multiple interactions with various interacting partners. Additionally, PTMs such as phosphorylation, can potentially affect the overall conformation of proteins [262, 263]. Previous lab experiments have shown that the mutation of Ser468 to alanine can change its interaction with COMMD1 and Cul-2 [182]. Thereby it was interesting to test whether different well characterized p65 phosphorylation sites (Ser276, Ser468 and Ser536) have the ability to affect p65 conformation. Those three serine residues are located in different regions of p65: Ser276 is located within the RHD while Ser486 and Ser536 are in the C-terminal TAD, as schematically shown in figure 1.7.

Kinases and signaling pathways leading to their phosphorylation are well known and extensively studied [93, 154, 158, 170, 173, 178, 179, 182].

To measure the conformational change of the whole p65 protein, limited proteolysis assays were performed. The assay is based on the ability of proteolytic enzymes to catalyse the hydrolysis of peptide bonds and to cleave proteins only at accessible residues at the protein surface. Proteases trypsin and thermolysin were employed for this assay. Trypsin cleaves at the C-terminus of the basic residues lysine and arginine, while thermolysin cleaves peptide bonds containing a hydrophobic amino acid, preferentially leucine or phenylalanine (Fig. 3.2A). HEK-293T cells were transiently transfected to express low amounts of either HA-tagged wild type p65, its non-phosphorylatable (serine to alanine) or phospho-mimetic (serine to glutamic acid) mutants (Fig. 3.2B). Whole cell extracts were prepared and digested with limited amounts of trypsin or thermolysin to assure that proteins are cleaved only partially. The fragmentation of p65 was monitored by Western blotting using a cocktail of two highly specific p65 antibodies recognizing the RHD (F6 antibody) and the TAD of p65 (C20 antibody). Immunoblotting showed the difference in digestion patterns between the wild type p65 and its mutated forms. Also, limited proteolysis of each p65 phospho-mutant resulted in different cleavage pattern of p65 when compared to each other. All together, proteolysis assays showed that the phosphorylation status of Ser276, Ser468 or Ser536 have an influence on p65 conformation, suggesting that phosphorylation contributes to the dynamic state of p65 conformation.

**A**



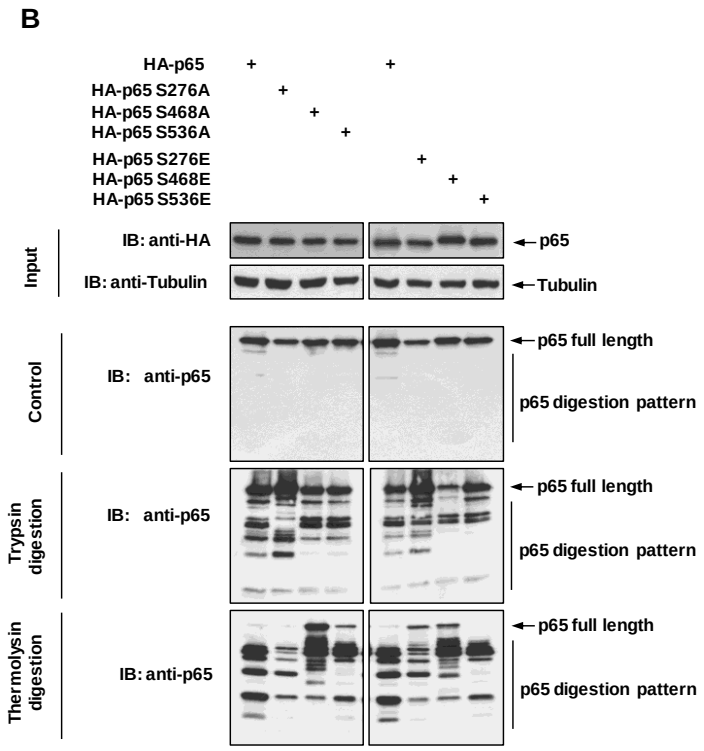



**Figure 3.2 Phosphorylation regulates structural flexibility of NF-κB p65.** (A) Hydrolysis of peptide bonds by trypsin and thermolysin. X, any residue. (B) HEK-293T cells were transiently transfected to express HA-tagged wild type p65 and its point-mutated versions where Ser276, Ser468 or Ser536 were changed to alanine or glutamic acid. Cells were lysed in NP-40 buffer. The lysate was digested with trypsin or thermolysine while an aliquot was just heated at 37˚C and served as a control. 10% of the lysate was used for the input control. Samples were analysed by SDS-PAGE and digestion patterns were revealed by immunoblotting (IB) with a mixture of two p65-specific antibodies, F6 and C20 (anti-p65). The input was analysed with indicated antibodies.

### 3.1.2 Individual phosphorylation sites control regulatory and degradative ubiquitination of p65

It has been previously shown that p65 phosphorylation has an impact on its ubiquitination [176, 182, 183, 210]. NF-κB p65 is modified by Lys48-polyUb, promoting its proteasome-mediated degradation [182, 209, 210, 212], but p65 can be also modified by non-degradative ubiquitination including Lys63-polyUb [201].

As the phosphorylation status of Ser276, Ser468 or Ser536 has an influence on p65 conformation, their impact on p65 ubiquitination was investigated. HEK-293T cells were transiently transfected to express low amounts of wild type p65 and its phospho-deficient mutants alone or in combination with 6 x His-tagged Ub (His-Ub). Ubiquitinated p65 proteins were purified on Ni-NTA beads and analysed by Western blotting (Fig. 3.3A). The C-terminal phosphorylation sites Ser468 and Ser536 showed a big impact on p65 ubiquitination as mutation of serine to alanine resulted in almost complete loss of Ub attachment to p65. Mutation of Ser276 only partially impaired p65 ubiquitination.

To test whether the tested phosphosites affect degradative ubiquitination of p65, cells were transfected to coexpress p65 or the indicated serine to alanine mutants with His-Ub (Fig. 3.3B). Cells were left untreated or were treated with the proteasome inhibitor MG132.

Inhibition of proteasomes additionally allows the accumulation and detection of p65 modified by Lys48-polyUb chains. Upon MG132 treatment, wild type p65 and p65 Ser276A mutant showed elevated levels of ubiquitination (Fig. 3.3B, lanes 1-4). In contrast, the intensity of p65 S468A and p65 S536A ubiquitination stayed unchanged, emphasizing the role of two C-terminal serine residues in the regulation of degradative ubiquitination (Fig. 3.3B, lanes 5-8). These results suggest that the control of regulatory and degradative p65 ubiquitination is attributable to phosphorylation of Ser468 and Ser536.

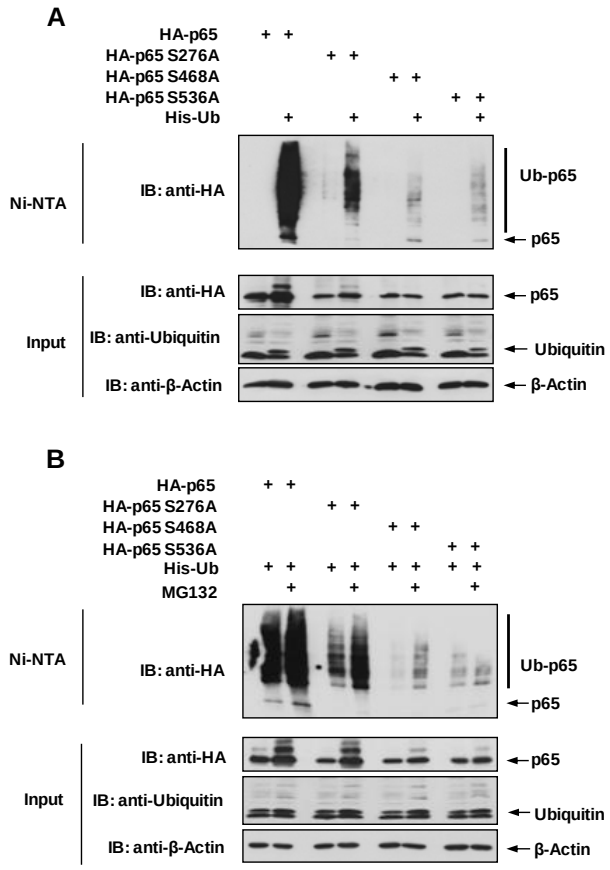



**Figure 3.3 Phosphorylation at Ser468 and Ser536 controls regulatory and degradative ubiquitination of p65.** (A) HA-tagged wild type p65 or p65 phosphorylation-deficient mutants were expressed in HEK-293T cells alone or together with His-Ub. The input fraction was lysed in 1x SDS lysis buffer, while the remaining 80% of cells were lysed in Ni-NTA lysis buffer. Ubiquitinated proteins were purified on Ni-NTA beads. Equal amounts of protein were separated by SDS-PAGE and analysed by immunoblotting with indicated antibodies. The presence of ubiquitinated p65 was detected using an antibody against the HA-tag. (B) The experimental settings were as in (A) except that cells were left untreated or treated overnight with 1 μM of the proteasome inhibitor MG132. After lysis under denaturing conditions and incubation with Ni-NTA beads, the ubiquitinated p65 proteins were analysed by immunoblotting with anti-HA antibodies. Expression of all HA-tagged p65 variants and ubiquitin was confirmed in the input by immunoblotting with appropriate antibodies. Ub-p65, ubiquitinated p65.

Since p65 is permanently phosphorylated and dephosphorylated under physiological conditions in the absence of NF-κB-inducing signals [173, 178], the effect of constitutive p65 phosphorylation on its ubiquitination was also tested. HEK-293T cells were transfected to express wild type p65 or its phospho-mimetic mutants alone or in combination with His-Ub. Ubiquitinated p65 proteins were purified under denaturing conditions on Ni-NTA beads and the ubiquitination status of p65 was analysed by Western blotting (Fig. 3.4A). Phospho-mimicking mutants of three tested serine residues showed

impaired regulatory p65 ubiquitination in comparison to wild type p65. To detect the impact of permanent phosphorylation on degradative ubiquitination, cells expressing wild type p65 or the indicated p65 phospho-mimetic mutants were co-transfected with His-Ub and left untreated or treated with MG132 (Fig. 3.4B). Immunoblotting with anti-HA antibody showed that MG132 treatment merely increased p65 S468E ubiquitination level, indicating that permanent phosphorylation at Ser468 rather protects p65 from degradative ubiquitination.

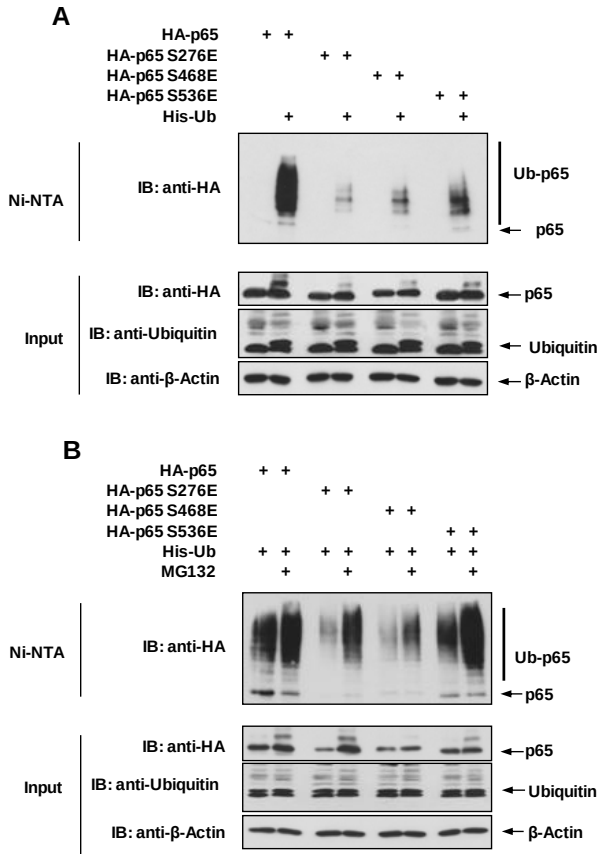



**Figure 3.4 An impact of constitutive p65 phosphorylation on regulatory and degradative ubiquitination.** (A) HA-tagged wild type p65 or p65 phospho-mimetic mutants were expressed in HEK-293T cells alone or together with His-Ub. Cells were lysed under denaturing conditions. An aliquot of cells was taken for an input and lysed in 1x SDS lysis buffer. The remaining cells were lysed in Ni-NTA lysis buffer, followed by purification of ubiquitinated proteins using Ni-NTA beads. Equal amounts of protein were separated by SDS-PAGE. The presence of ubiquitinated p65 was detected using anti-HA antibodies. The input fraction was immunoblotted with indicated antibodies. (B) The experimental settings were as in (A) except that cells were left untreated or treated overnight with the proteasome inhibitor MG132. After cell lysis under denaturing conditions and incubation with Ni-NTA beads, precipitated ubiquitinated p65 proteins were separated by SDS-PAGE and detected by immunoblotting with indicated antibodies.

In agreement with the limited proteolysis assays, these experiments show that the phosphorylation status of individual serine residues affects p65 conformation and consequently also p65 ubiquitination.

### 3.1.3 Cytokines induce a phosphorylation-dependent conformational change of nuclear p65

In order to detect the occurrence of p65 conformational change under physiological conditions, cells were stimulated with TNF to trigger activation of NF-κB. HEK-293 cells

were left unstimulated or stimulated with TNF for 15 min. Cytosolic and nuclear fractions were prepared and partially digested with trypsin. Immunoblotting with a cocktail of p65-specific antibodies showed no significant changes in the digestion patterns of cytosolic p65 (Fig. 3.5A). Interestingly, TNF stimulation induced a strongly altered digestion pattern of nuclear p65, as revealed by the occurrence of a prominent double band indicated as p65* (Fig. 3.6A). These results suggest that p65 undergoes a conformational rearrangement upon TNF stimulation.

It has been shown that NF-κB appears in the nucleus within 5 min and reaches its maximum level in the nucleus after 30 min of TNF stimulation. During that period IκBα levels rapidly decrease, but resynthesis of IκBα proteins occurs already after 60 min [69]. In order to see if the conformational change detected after 15 min of the TNF stimulation is transient and occurs independently of the p65 interaction with IκBα, the kinetics of TNF-triggered p65 refolding was analysed. HEK-239 cells were stimulated with TNF for 0, 30 or 90 min and nuclear extracts were digested with trypsin (Fig. 3.5B). Immunoblotting with p65-specific antibodies revealed the same fragmentation pattern of p65 after TNF stimulation for 30 or 90 min. The p65* double band occurred irrespectively whether IκBα was degraded or re-synthesized, implicating that the TNF-induced p65 conformational change was not transient and not dependent on interaction with IκBα.

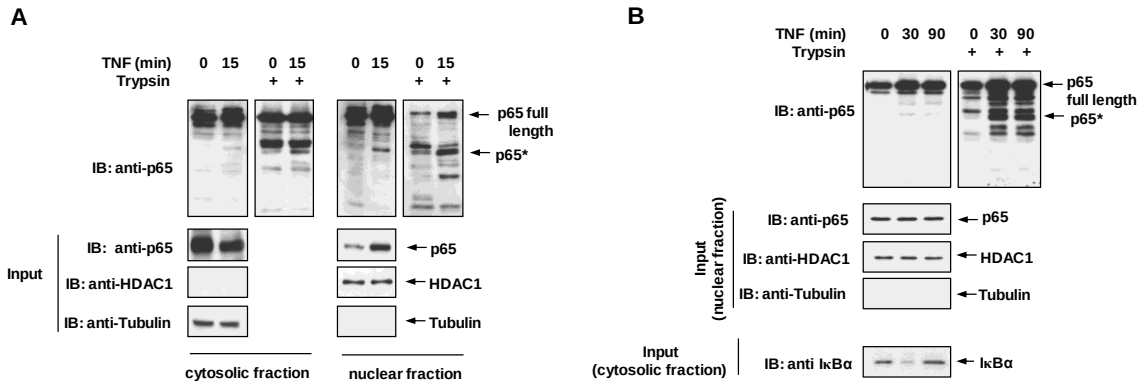



**Figure 3.5 TNF induces a conformational change of nuclear p65.** (A) HEK-293 cells were left untreated or stimulated for 15 min with TNF (10 ng/ml). Cells were fractionated into cytosolic and nuclear extracts. Extracts remained untreated or digested with trypsin and analysed by immunoblotting with p65-specific antibodies. The position of a prominent double band that occurs after TNF stimulation in nuclear extracts is indicated as p65*. The lower part shows input controls and ensures the purity of fractions, as revealed by the occurrence of marker proteins for the nucleus (HDAC1) and the cytosol (Tubulin). (B) HEK-293 cells were stimulated with TNF for 0, 30 or 90 min. Nuclear extracts were prepared and left undigested or digested with trypsin. Samples were analysed as in (A).

It was interesting to test whether the cytokine IL-1 induces the same conformational rearrangement as TNF. As HEK-293 cells hardly respond to IL-1, HEK-293 cells expressing the IL-1 receptor (HEK-293IL-1R) were used for these experiments. Cells were stimulated with TNF or IL-1 for 0 or 15 min. Cytosolic and nuclear extracts were prepared and digested with trypsin (Fig. 3.6A). Stimulation with IL-1 induced the same pattern of p65 fragmentation when compared to TNF-treated nuclear p65. This suggests that both cytokines induce the same configuration of p65. The double p65* marker band was detected after 90 min of IL-1 stimulation, confirming that rearrangement of nuclear p65 is not a transient event (Fig. 3.6B).

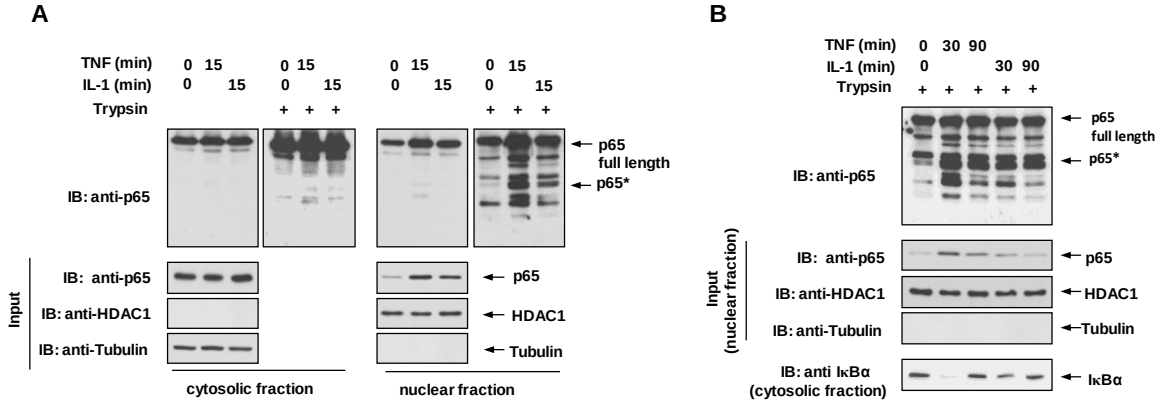



**Figure 3.6 Comparison between IL-1- and TNF-induced conformational changes of nuclear p65.** (A) HEK-293IL-1R cells were left untreated or stimulated for 15 min with TNF (10 ng/ml) or IL-1 (10 ng/ml). Cells were fractionated into cytosolic and nuclear extracts. These extracts remained untreated or partially digested with trypsin. Fragmentation of p65 was analysed by immunoblotting with p65-specific antibodies. An aliquot was used to ensure the purity of lysates (Input). (B) HEK-293IL-1R cells were stimulated with TNF or IL-1 for 0, 30 or 90 min. Nuclear extracts were prepared and digested with trypsin. Samples were analysed as in (A).

The relative contribution of phosphorylation for the cytokine-induced conformational change of nuclear p65 was tested. For this purpose HEK-293 cells were treated with calyculin A to induce phosphorylation of endogenous p65 by strong inhibition of serine/threonine protein phosphatases [177, 178, 185, 264]. Also treatment with calyculin A induced the appearance of the p65* double band, as revealed by limited proteolysis assays of nuclear extracts (Fig. 3.7A). To address the contribution of individual phosphorylation sites on cytokine-induced p65 folding, mouse embryonic fibroblasts lacking the p65 gene (p65-/- MEFs) were reconstituted with lentiviral constructs to stably express HA-tagged wild type p65 or its phospho-deficient mutants (p65 S468A or p65 S536A). Cells were stimulated with TNF and nuclear extracts were tested for p65

conformational rearrangements by limited proteolysis experiments. Immunoblotting with the anti-HA antibody detected TNF-induced changes in the cleavage pattern of wild type p65, while mutation of the individual phosphorylation sites caused only prominent changes in digestion patterns and intensities of p65 fragments (Fig. 3.7B and C, lanes 2 and 4). Although these experiments do not show the contribution of one single phosphorylation site for the complete switch, they support the finding that phosphorylations mediate folding of p65.

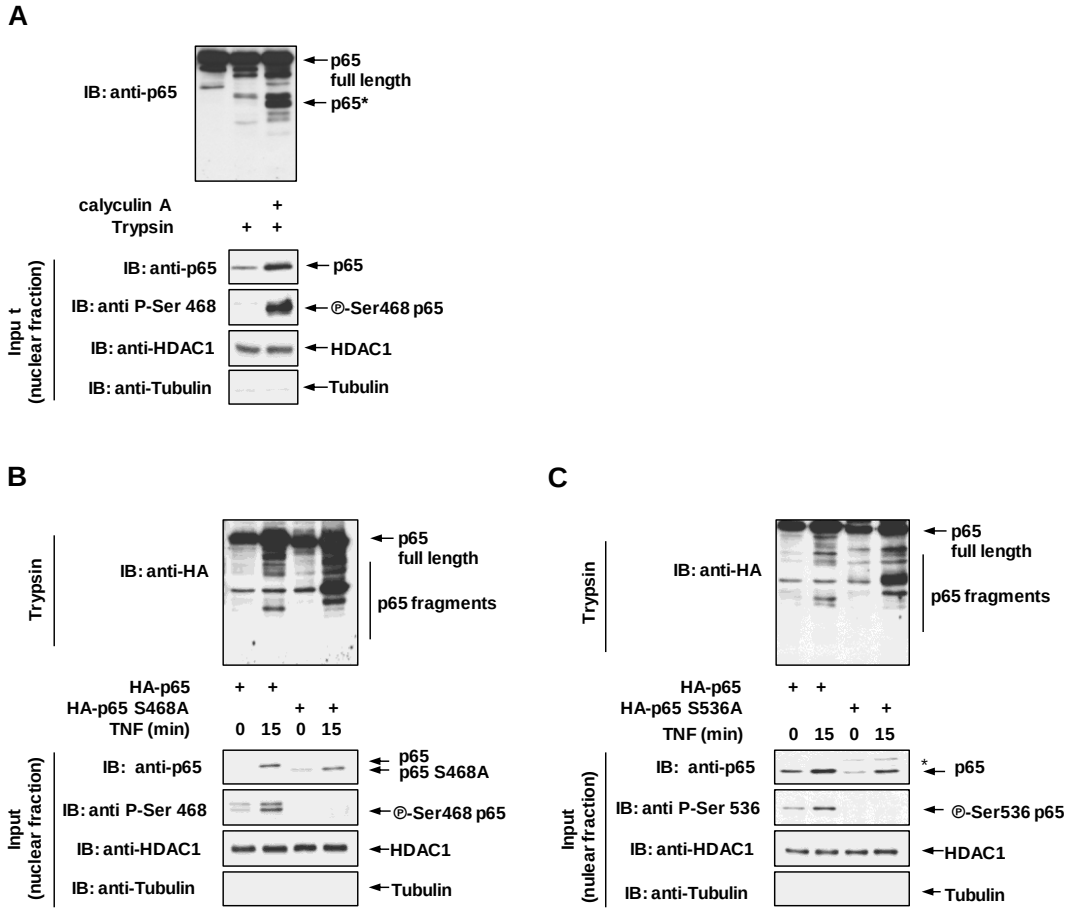



**Figure 3.7 TNF-induced structural rearrangement of nuclear p65 is mediated by phosphorylations.** (A) HEK-293 cells were untreated or treated with calyculin A (50 nM) for 30 min and nuclear extracts were subjected to a limited digestion assay with trypsin. Fragments were analysed by immunoblotting with a cocktail of anti-p65-specific antibodies. The position of the p65* double band is shown. The lower part shows the input controls and ensures the purity of fractions, as revealed by the occurrence of marker proteins for the nucleus (HDAC1) and the cytosol (Tubulin). (B) p65-/- MEFs were reconstituted with HA-tagged wild type p65 or p65 S468A. Cells were stimulated with TNF (10 ng/ml) for 15 min and nuclear extracts were subjected to a limited protease digestion assay. Fragments were analysed by immunoblotting with the anti-HA antibody. (C) p65-/- MEFs were reconstituted with HA-tagged wild type p65 or p65 S536A mutant. The experiment was done as in (B). Digestion patterns of wild type p65 were compared to that of the mutants. * indicates a non-specific band.

### 3.1.4 The TNF-induced NF-κB p65 structural rearrangement unmasks an epitope localised in the TAD2 subdomain

The cytokine-induced conformational change of nuclear p65 was tested by an alternative approach. Seven p65-specific antibodies were tested for their ability to immunoprecipitate p65 in its active conformation after TNF stimulation (Fig. 3.8). Each tested antibody recognizes a different p65 epitope, as schematically illustrated in green and grey in figure 3.8A. The antibodies F6 and C20 were used as a control (Fig. 3.8A, represented in red). HEK-293 cells were left unstimulated or exposed to TNF for 15 min after which the nuclear extract was prepared and pre-cleared with agarose beads. Immunoprecipitation was performed with antibodies indicated in figure 3.8A and corresponding control IgG antibodies. Eluted p65 proteins were detected by immunoblotting with the p65-specific antibody C20 (Fig. 3.8B).

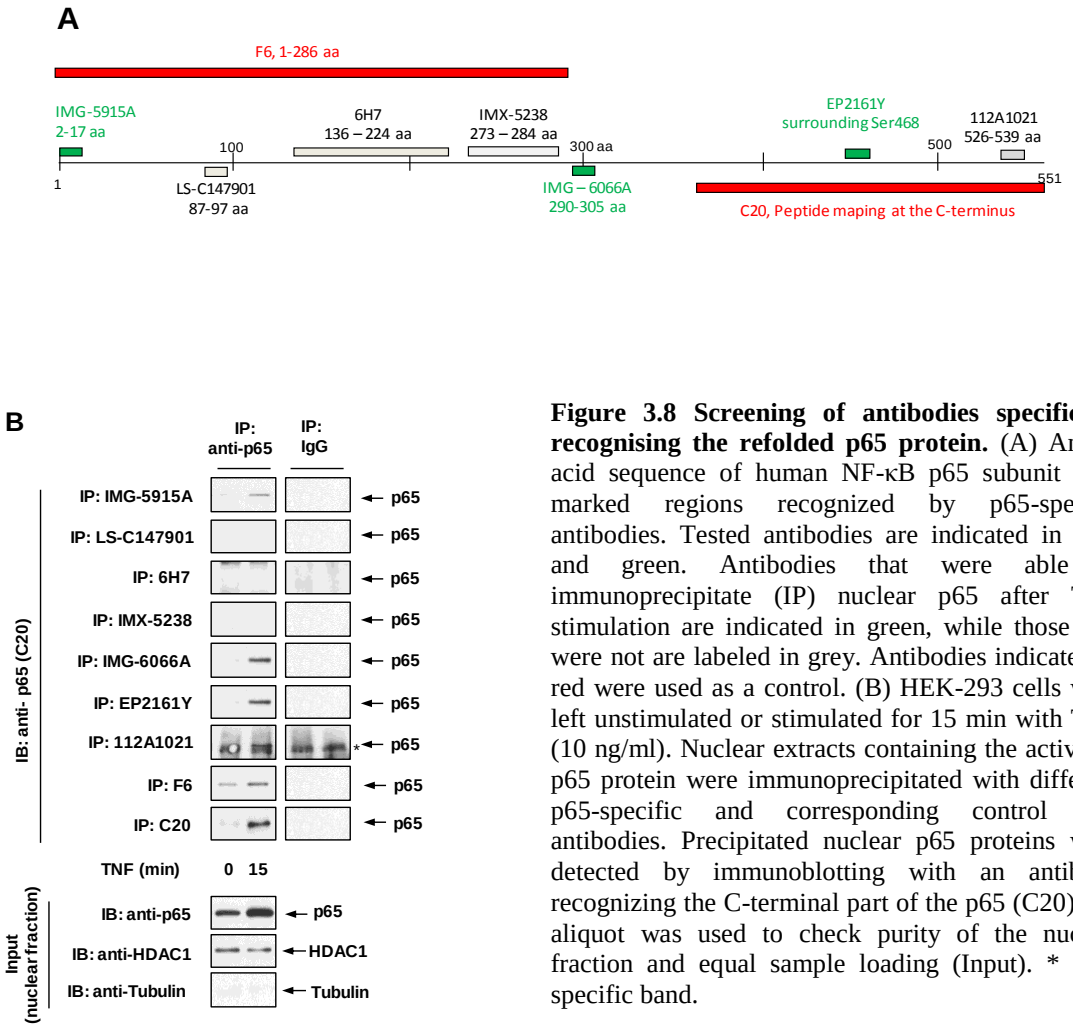



**Figure 3.8 Screening of antibodies specifically recognising the refolded p65 protein.** (A) Amino acid sequence of human NF-κB p65 subunit with marked regions recognized by p65-specific antibodies. Tested antibodies are indicated in gray and green. Antibodies that were able to immunoprecipitate (IP) nuclear p65 after TNF stimulation are indicated in green, while those that were not are labeled in grey. Antibodies indicated in red were used as a control. (B) HEK-293 cells were left unstimulated or stimulated for 15 min with TNF (10 ng/ml). Nuclear extracts containing the activated p65 protein were immunoprecipitated with different p65-specific and corresponding control IgG antibodies. Precipitated nuclear p65 proteins were detected by immunoblotting with an antibody recognizing the C-terminal part of the p65 (C20). An aliquot was used to check purity of the nuclear fraction and equal sample loading (Input). * non-specific band.

These experiments showed that an antibody recognizing a region surrounding Ser468 (Abcam EP2161Y) was able to immunoprecipitate the active, nuclear form of p65. An antibody recognizing the NLS of p65 (Imgenex IMG-6066A) was also able to immunoprecipitate TNF-activated p65 as reported previously [265]. However, the specificity of this antibody relies on a different principle, as it can only recognize the NLS of p65 when IκBα is degraded.

### 3.1.5 Phosphorylation-dependent changes of NF-κB p65 conformation regulate p65 interactions with transcriptional cofactors

Several studies have shown that phosphorylation of p65 governs the outcome of NF-κB responses through differential interaction with various enzymes [154, 166, 177, 182, 183, 266]. Therefore it was interesting to investigate the impact of p65 phosphorylation-dependent structural changes on protein-protein interactions. Phosphorylation at the N-terminal Ser276 and the C-terminal Ser468 leads to different rearrangement of p65 (Fig. 3.2B). Therefore, coimmunoprecipitation experiments were performed to compare the binding of known p65 interactors to wild type p65 and phospho-mimetic p65 S468E and p65 S276E mutants. The interaction with the p65 interacting partner CBP was tested since it can interact with both the N- and C-terminal half of the p65 [154]. Another tested interaction partner was the NF-κB corepressor MYBBP1a (Myb-binding protein 1a), as this protein interacts with the C-terminal half of p65 [267, 268]. Cells were transfected to express p65 variants alone or together with CBP, MYBBP1a or the NF-κB p50 subunit. NF-κB interacting partners were immunoprecipitated with anti-GFP or anti-Flag antibodies and the amount of a bound p65 protein was revealed by immunoblotting with anti-HA antibodies. Permanent phosphorylation at Ser468 reduced the interaction with CBP and MYBBP1a proteins and also led to reduced binding of p65 to NF-κB subunit p50 (Fig. 3.9A). In contrast to Ser468, permanent phosphorylation at Ser276 did not change the ability of p65 to interact with the tested interacting partners (Fig. 3.9B). These coimmunoprecipitation experiments implicate that a phosphorylation-induced conformational change affects the association of p65 with transcriptional coactivators and corepressors, as exemplified for p65 phospho-mimetic Ser468 and Ser276 mutants. Additionally, these experiments emphasize the role of Ser468 for the conformation of p65 and its affinity to interaction partners.

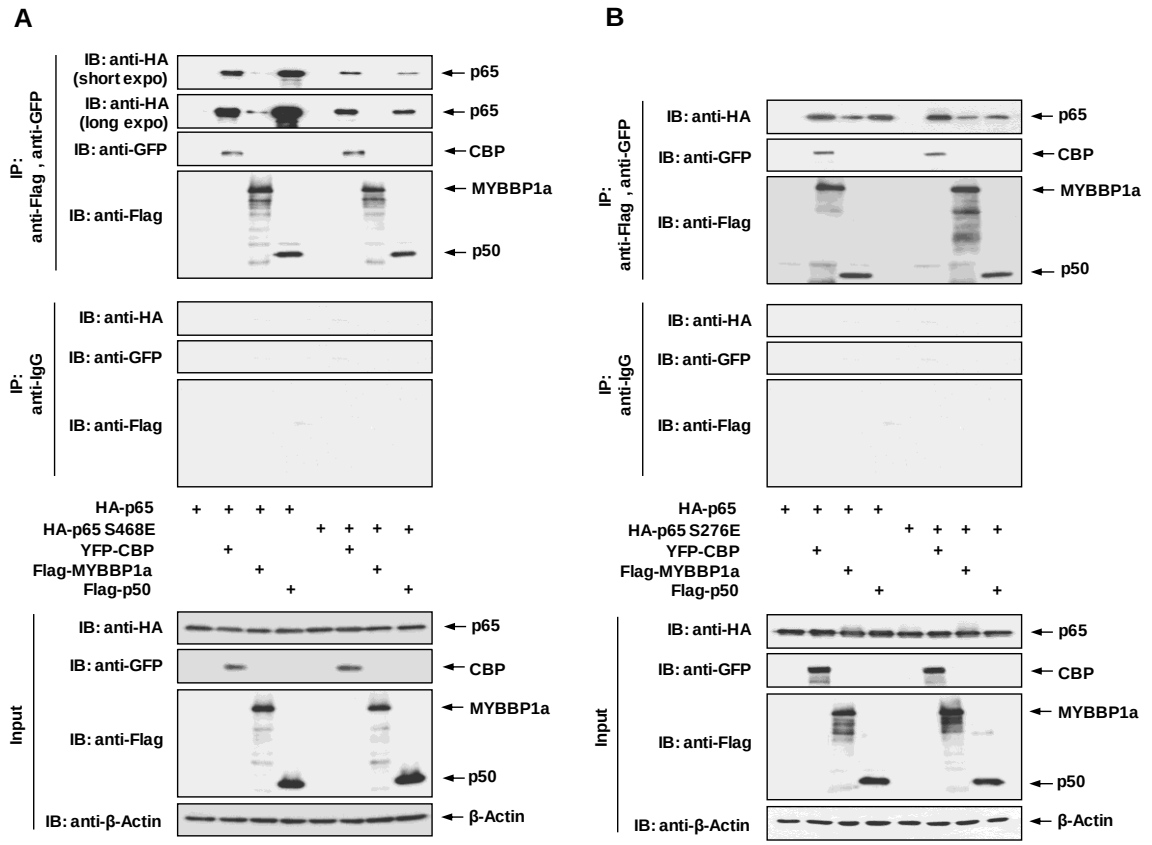



**Figure 3.9 NF-κB p65 Ser468 phosphorlylation controls protein-protein interactions.** (A) HA-tagged wild type p65 or the p65 S468E mutant were expressed alone or together with indicated proteins. Two days later, whole cell lysates were immunoprecipitated with anti-Flag, anti-GFP antibodies or control IgG antibodies. Immunoprecipitated proteins were detected by Western blotting with indicated antibodies. Two different exposures are displayed for the coprecipitated p65 protein to allow the visualization of differences for binding of the MYBBP1a protein. (B) HA-tagged wild type p65 or the p65 S276E phospho-mutant were expressed alone or together with various indicated proteins in HEK-293T cells. Immunoprecipitation and detection of the proteins was done as in (A).

## 3.2. Regulation of p65 refolding and activity by chaperones

### 3.2.1 NF-κB p65 physically interacts with Hsc/Hsp70 and Hsp90α

The C-terminal part of p65 is unstructured and only under certain experimental conditions it adopts a defined secondary structure [180]. Chaperones are defined as proteins that interact, stabilize and/or help another protein to acquire its functionally active conformation [222, 269]. Thus, it was interesting to investigate whether the TNF-induced nuclear p65 conformational change is regulated by chaperones.

First, a physical interaction between p65 and constitutively expressed Hsc70, stress-inducible expressed Hsp70 or Hsp90α was tested (Fig. 3.10). HEK-293T cells were transiently transfected to express moderate amounts of epitope tagged wild type p65 alone

or together with various Hsps or p50 as a positive control. Coimmunoprecipitation experiments showed an interaction between p65 and Hsc70 or Hsp70, which is in line with previous studies [242, 257]. The interaction with Hsp90α was not observed (Fig. 3.10A). Hsp90 interacts with its client proteins with a weak affinity and only transiently [229]. To test a potential interaction between p65 and Hsp90α by a more sensitive approach, coimmunoprecipitation experiments were performed in the presence of the membrane permeable cross-linker DTBP, which reversibly cross-links and stabilizes protein-protein interactions which occur in close proximity. Cells were transfected to express HA-tagged p65 alone or together with Flag-tagged Hsp90α. Before lysis, cells were incubated with vehicle or DTBP. Coimmunoprecipitation detected an interaction between p65 and Hsp90α only after the treatment with DTBP (Fig. 3.10B, lane 3), revealing that this interaction is rather weak.

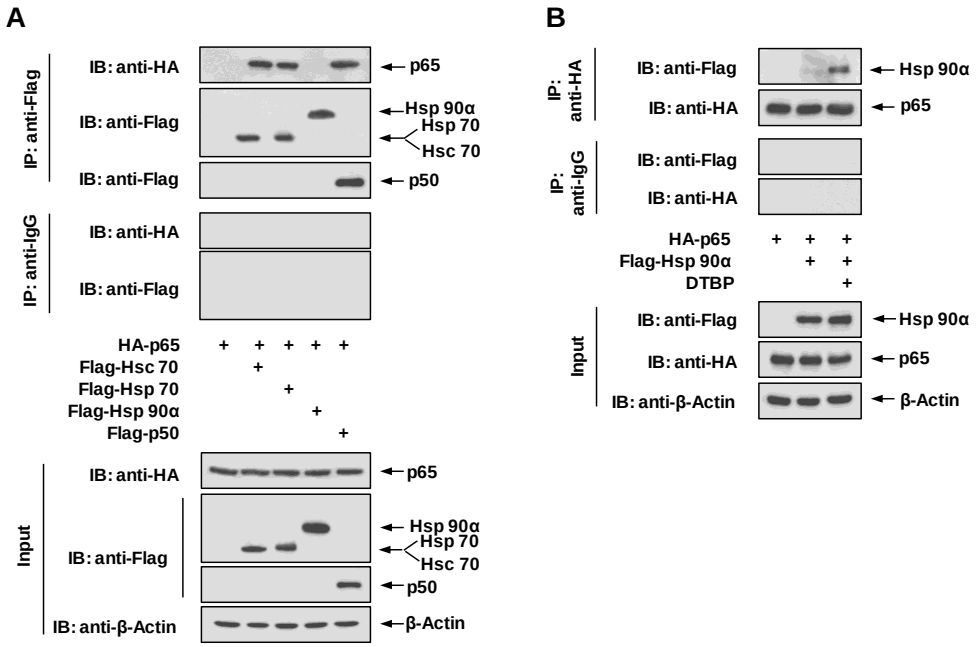



**Figure 3.10 p65 physically interacts with Hsps.** (A) HEK-293T cells were transiently transfected to express HA-tagged p65 together with the Flag-tagged Hsc70, Hsp70 or Hsp90α. Coexpression with Flag-tagged p50 served as a positive control. Cells were lysed in NP-40 buffer and an aliquot was used for the analysis of protein expression (Input). The remaining lysate was used for immunoprecipitation with anti-Flag or control IgG antibodies. Precipitated proteins were detected by immunoblotting with indicated antibodies. (B) HEK-293T cells were transfected with indicated plasmids and incubated with the cross-linker DTBP. After NP-40 lysis, an aliquot was used for the input control, while the rest of the lysate was immunopreciptated with either anti-Flag or control IgG antibodies; precipitated proteins were detected by immunoblotting with indicated antibodies.

The proinflammatory cytokine TNF is produced under various stress conditions and leads to increased Hsp70 expression in cardiac myocytes [270]. It has been demonstrated that

upregulation of Hsc70 and Hsp70 results in increased nuclear translocation of p65 [242, 271]. Therefore, the association between endogenous p65 and Hsp70 was tested in TNF-stimulated cells (Fig. 3.11). HEK-293 cells were stimulated with TNF and Hsp70 was immunoprecipitated from whole cell extracts using an Hsp70-specific antibody. Immunoblotting with an anti-p65 antibody detected the interaction between endogenous p65 and Hsp70. This interaction occurred independently of TNF stimulation (Fig. 3.11A). Interaction between endogenous p65 and Hsp70 was detectable in the nuclear fraction only after TNF treatment (Fig. 3.11B).

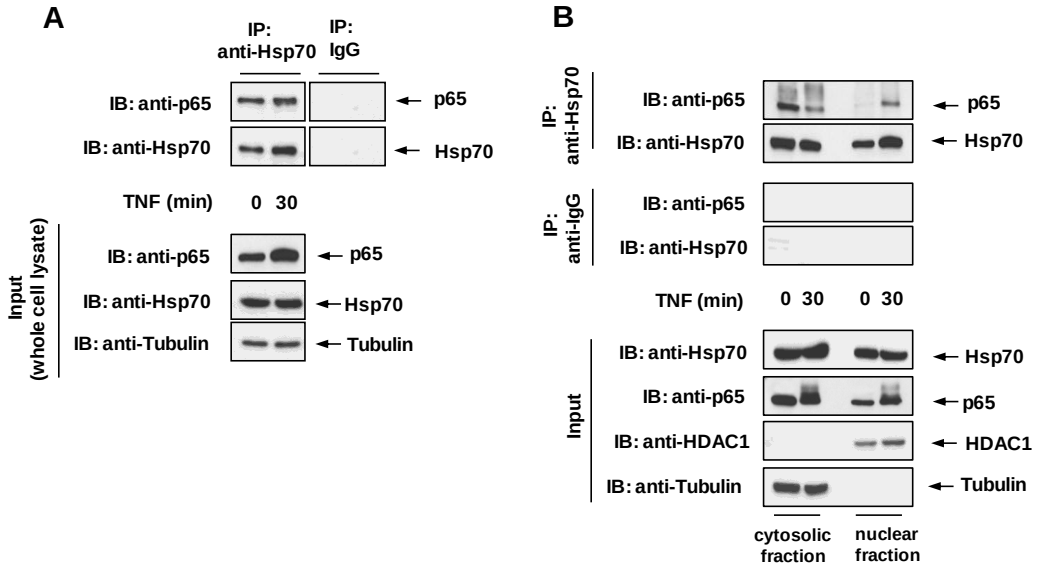



**Figure 3.11 Endogenous p65 and Hsp70 constitutively intaract.** (A) HEK-293 cells were left untreated or treated with TNF (10 ng/ml) for 30 min. After lysis with NP-40 lysis buffer and immunoprecipitation with anti-Hsp70 antibodies and control IgG antibodies, precipitated proteins were separated with SDS-PAGE and analysed by Western blotting. An aliquot of the lysate (10%) was used to confirm expression of proteins. (B) HEK-293 cells were left unstimulated or stimulated with TNF (10 ng/ml) for 30 min. After the subcellular fractionation into cytosolic and nuclear extracts, these were used to immunoprecipitate Hsp70. The binding of p65 was analysed by immunoblotting, an aliquot was used as a loading control (Input).

### 3.2.2 Phosphorylation-dependent structural changes of p65 regulate p65 interaction with Hsps

The impact of permanent phosphorylation at Ser468 and Ser276 on the ability of p65 to bind Hsp70 and Hsp90 was tested (Fig. 3.12). Cells were transfected to express wild type p65 or its phospho-mimicking versions alone or together with Hsp70 or Hsp90. To test the interaction with Hsp90, cells were incubated with DTBP prior to lysis. Immunoprecipitation was performed using the anti-Flag antibody. Coimmunoprecipitation

of p65 with Hsp70 (Fig. 3.12A/C) or Hsp90 (Fig. 3.12B/D) showed that permanent Ser468 phosphorylation strongly diminished the association with Hsp90, while it slightly enhanced the interaction of p65 with Hsp70. Permanent phosphorylation at Ser276 slightly reduced the interaction of p65 with both tested chaperones. These protein-protein interaction studies support the idea that the phosphorylation-dependent p65 conformational change affects affinities of p65 to its interaction partners.

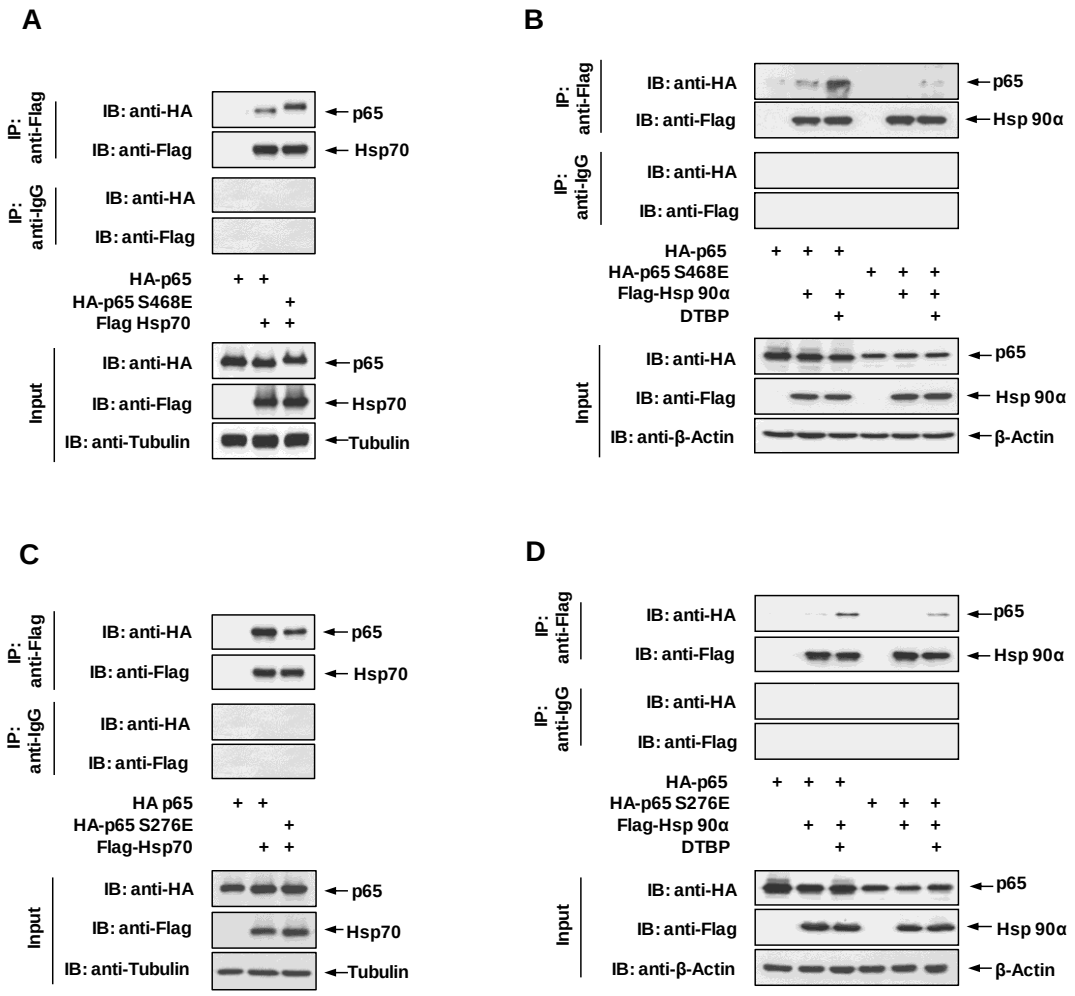



**Figure 3.12 Permanent Ser468-phosphorylation affects the interaction of p65 with Hsps.** (A) HA-tagged wild type p65 or its phospho-mimetic mutant HA-p65 S468E were transfected alone or together with Flag-tagged Hsp70. HEK-293T cells were lysed in NP-40 buffer. An aliquot was analysed for correct protein expression (Input). Immunoprecipitation was performed using an anti-Flag antibody or a control IgG antibody. Precipitated proteins and input samples were analysed by SDS-PAGE and Western blotting using appropriate antibodies. (B) HEK-293T cells were transfected to express epitope-tagged versions of p65 and Hsp90α as shown and treated with vehicle or the cross-linker DTBP. Cells were lysed and subjected to immunoprecipitation using an anti-Flag antibody. An aliquot was used for input control. The precipitates and input controls were analysed by immunoblotting with indicated antibodies**.** (C) and (D) Experiments were done as in (A) and (B) respectively, except that the HA-p65 S276E mutant was used.

### 3.2.3 Chaperones contribute to the control of the TNF-induced p65 refolding

A substantial fraction of all proteins in eukaryotic cells adopt a folded conformation only after interaction with a cognate binding partner [222]. In order to gain a functional conformation, some proteins need further interaction with the Hsp90 and/or Hsp70 [229, 231]. To address whether Hsps contribute to basal p65 conformation, Hsps were pharmacologically inhibited. Hsp90 was inhibited by GA and Hsc/Hsp70 by the use of VER. Both inhibitors block the ATPase activity of Hsps, thereby inhibiting their chaperone activity [223, 247, 272, 273]. HEK-293 cells were incubated with vehicle, GA and VER for 17 h and whole cell lysates were subjected to limited proteolysis assays. Immunoblotting with p65-specific antibodies, revealed no difference in p65 digestion patterns after the inhibition of Hsp90 or Hsc/Hsp70 in comparison to untreated cells (Fig. 3.13).

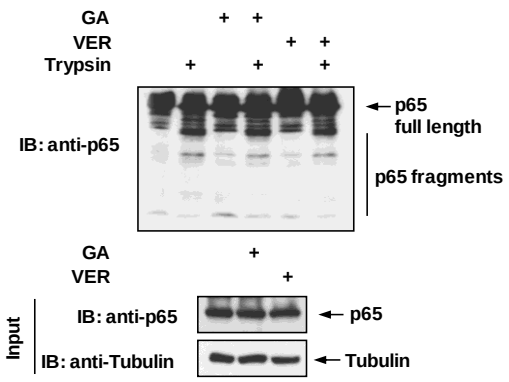



**Figure 3.13 Chaperones have no influence on basal conformation of p65.** HEK-293 cells were left untreated or treated with inhibitors GA (1 μM) or VER (30 μM). Whole cell lysate was prepared and 10% was used for the input control. The rest of the lysate was used for the limited proteolysis assay. Extracts were left untreated or treated with trypsin. p65 fragments and input controls were separated by SDS-PAGE and analysed by immunoblotting with p65-specific and anti-Tubulin antibodies.

With the aim to test the role of Hsp70 and Hsp90 in folding of newly synthesized p65, it was necessary to induce the degradation of p65 proteins that were present inside cells. For that aim, HeLa cells were first stimulated with a TNF pulse for 15 min, followed by blockage of new protein synthesis by CHX for indicated periods. As CHX prevents the resynthesis of the eliminated protein and thus allows the detection of TNF-induced p65 diminishment (Fig. 3.14A). After the CHX treatment for 24 h which caused almost complete loss of p65, cells were washed with PBS to remove the reversible translation inhibitor CHX [274, 275] and to allow synthesis of new proteins. After an incubation period of 3 h, cells were treated with vehicle, VER and GA for the next 17 h in order to detect a possible contribution of Hsps on folding of newly synthesized p65 (Fig. 3.14B). Limited proteolysis assays followed by immunoblotting did not show a significant

difference in the digestion pattern of *de novo* synthesized p65 protein, indicating that Hsps are not required for p65 folding in unstimulated cells.

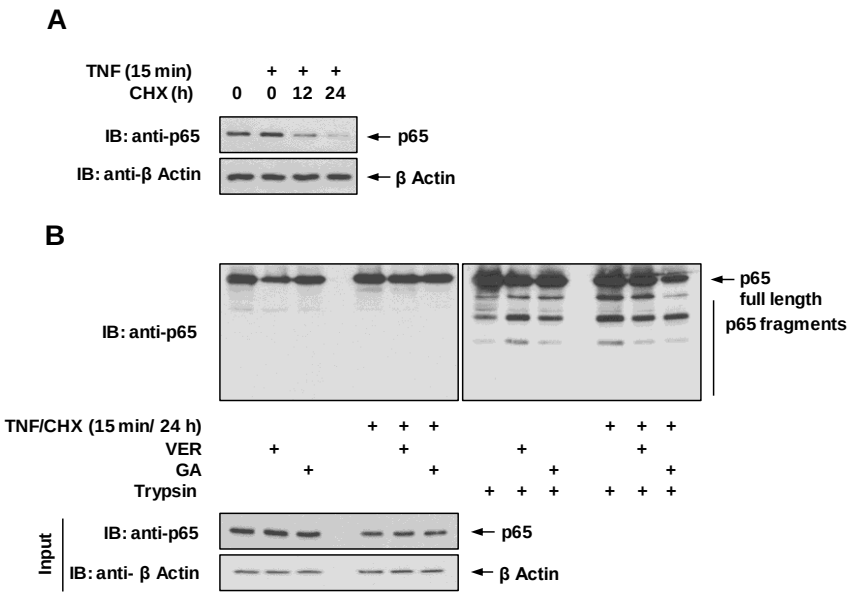



**Figure 3.14 Chaperones have no influence on the conformation of a newly synthesized p65.** (A) HeLa cells were stimulated with a pulse of TNF (10 ng/ml) for 15 min. The stimulus was removed by washing cells twice with pre-warmed PBS followed by treatment with CHX (10 µg/ml) for indicated periods. Cells were lysed in NP-40 buffer and the stability of p65 was detected by SDS-PAGE followed by immunoblotting with anti-p65 and anti-β-Actin specific antibodies. (B) HeLa cells were stimulated with a pulse of TNF (10 ng/ml) for 15 min. After the washout of stimulus, cells were treated with CHX for 24 h (10 µg/ml) to prevent resynthesis of the proteosomally degraded p65. Cells were washed with pre-warmed PBS and incubated for 3 h with the complete medium. After the incubation period, cells were treated with vehicle, VER (30 µM) or GA (1 µM) for the next 17 h. Cells were lysed with NP-40 buffer. Extracts were digested with trypsin or just heated. Digestion patterns were revealed by SDS-PAGE and analysed by Western blotting for p65 and β-Actin. Total cell extracts were analysed by immunoblotting for the occurrence of p65 and β-Actin using specific antibodies (Input).

To reveal a contribution of Hsps as chaperones for the TNF-induced conformational change of nuclear p65, cells were first treated with vehicle, GA and VER for 17 h and then stimulated with TNF for 15 min prior to cell lysis. Nuclear extracts were prepared and analysed for the p65 conformational change by limited digestion with trypsin or thermolysin (Fig. 3.15). TNF-induced refolding occurred also in the presence of Hsps inhibitors, as concluded by detection of the characteristic p65* double band. However, the characteristic fragments of cleaved p65 were more intensive in the presence of VER (Fig. 3.15, lane 4). These results suggest that the Hsc/Hsp70 system is not essential for the p65 refolding but rather participate in the control of the fidelity and/or kinetics of TNF-induced p65 refolding.

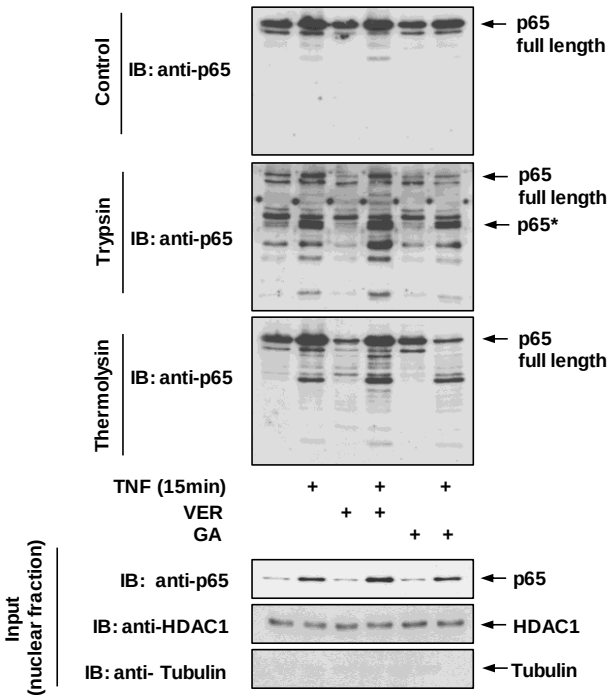



**Figure 3.15 Hsc/Hsp70 system contributes to the TNF-induced nuclear p65 conformational rearrangement.** HEK-293 cells were left untreated or preincubated with VER (1μM) or GA (30 μM) for 17 h, followed by TNF stimulation (10 ng/ml) for 15 min. Nuclear extracts were digested with trypsin or thermolysin as shown. The fragmentation of p65 was revealed by Western blotting with p65-specific antibodies. The input material was analysed by immunoblotting with indicated antibodies.

### 3.2.4 Hsc/Hsp70 proteins control the level of p65 PTMs and p65-mediated gene expression

Hsps control the folding and biological activity of kinases and TFs [276] and are therefore involved in the regulation of signal transduction [231, 277]. Hsp70 and Hsp90 control NF-κB activity through the regulation of the IKK complex [248, 249, 255]. Also, the interaction between Hsc70 and Hsp70 with NF-κB p65 subunit was described [242, 257]. Therefore the role of Hsc/Hsp70 and Hsp90 proteins for the induction of p65 phosphorylation, acetylation and ubiquitination was tested.

#### 3.2.4.1 Hsc/Hsp70 proteins regulate p65 PTMs

The influence of Hsps on TNF-induced p65 phosphorylation was tested. HEK-293 cells were incubated with GA or VER for 17 h, followed by stimulation with TNF for 30 min in order to induce NF-κB activation (Fig. 3.16). Upon VER treatment the level of TNF-induced p65 phosphorylation at Ser468 and Ser536 was increased. Inhibition of Hsp90 caused a reduction of p65 phosphorylation at both serine residues.

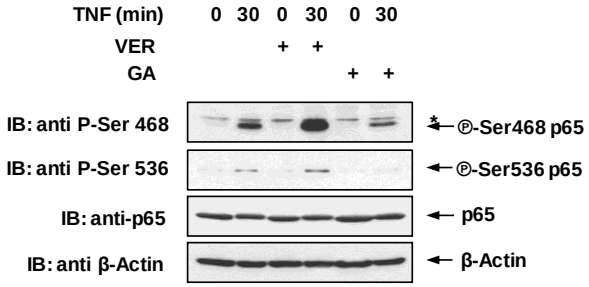



**Figure 3.16 Hsps regulate p65 phosphorylation.** HEK-293 cells were incubated with vehicle, GA or VER overnight. Cells were left unstimulated or stimulated with TNF (10 ng/ml) for 30 min prior to lysis. Whole cell lysates were separated by SDS-PAGE and analysed by immunoblotting for the occurrence of p65 phosphorylation. *non-specific band.

To reveal the impact of Hsps on p65 acetylation, CBP-triggered acetylation of p65 was measured in the presence of GA and VER (Fig. 3.17A). HEK-293T cells were transiently transfected with Flag-tagged wild type p65 alone or together with YFP-tagged CBP. Two days post-transfection, cells were lysed and p65 was immunoprecipitated using anti-Flag antibodies. The level of acetylated p65 was revealed by immunoblotting with an antibody specific for acetylated lysine residues. Acetylation of p65 was strongly impaired in the presence of the Hsc/Hsp70 inhibitor, while GA treatment remained without a significant impact on this modification. Additionally, the level of induced p65 acetylation after over-expression of Hsp70 was measured. HEK-239T cells were transfected to coexpress Flag-tagged p65, the HA-tagged CBP-related acetyltranferase p300 or/and HA-tagged Hsp70. Immunoprecipitation was performed using the anti-Flag antibody. Overexpression of Hsp70 slightly enhanced the level of p65 acetylation supporting the finding that chaperone activity contributes to p65 acetylation (Fig. 3.17B, lane 2 and 4).

Also the level of basal p65 ubiquitination was investigated after Hsp inhibition. HEK-293T cells were transfected to express HA-p65 alone or together with His-Ub. The next day, cells were treated with vehicle, GA or VER for 17 h, followed by lysis under denaturing conditions and purification of ubiquitinated proteins by Ni-NTA beads. The ubiquitinated p65 was detected by immunoblotting with anti-HA antibodies (Fig. 3.18A). In comparison to untreated cells, VER treatment markedly reduced the level of p65 ubiquitination in comparison to untreated cells. The inhibition of Hsp90 did not show any effect on p65 ubiquitination. It was then interesting to test whether impaired p65 ubiquitination results in inhibition of TNF-induced p65 degradation. HeLa cells were stimulated with a pulse of TNF for 15 min and further incubated in the presence of CHX to prevent resynthesis of eliminated proteins. Cells were preincubated for 4 h with vehicle or VER before the TNF stimulation (Fig. 3.18B). Inhibition of Hsc/Hsp70 prevents p65 TNF-induced degradation, as revealed by immunoblotting with p65-specific antibodies.

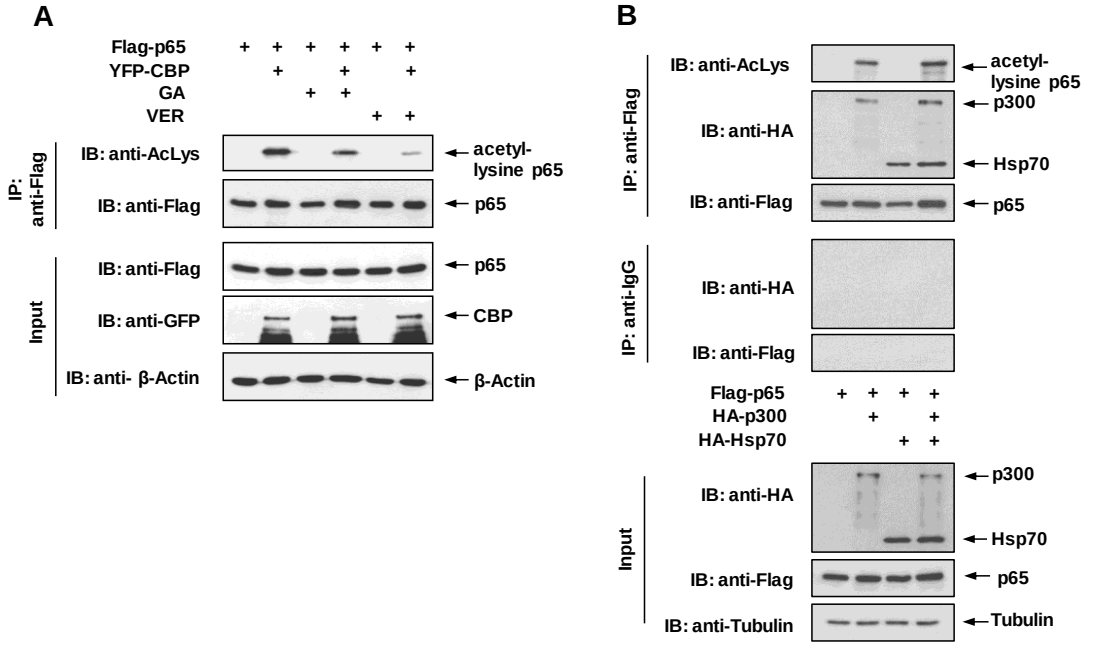



**Figure 3.17 The Hsc/Hsp70 system regulates p65 acetylation.** (A) Flag-tagged p65 was expressed in HEK-293T cells alone or together with the YFP-tagged acetyltransferase CBP and 17 h before cell lysis, cells were treated with vehicle, GA or VER. p65 was immunoprecipitated from the whole cell lysate by anti-Flag antibodies and the occurrence of p65 acetylation was detected by immunoblotting with an antibody that detects acetylated lysines. (B) Flag-tagged wild type p65 was transfected alone or together with HA-tagged p300 or/and Hsp70. Two days later, HEK-293T cells were lysed in NP-40 buffer. p65 was precipitated with an anti-Flag antibody. The amount of coprecipitated p300 or/and Hsp70 were detected by SDS-PAGE and Western blotting with appropriate antibodies. Acetylated p65 was detected with an acetyl-lysine antibody.

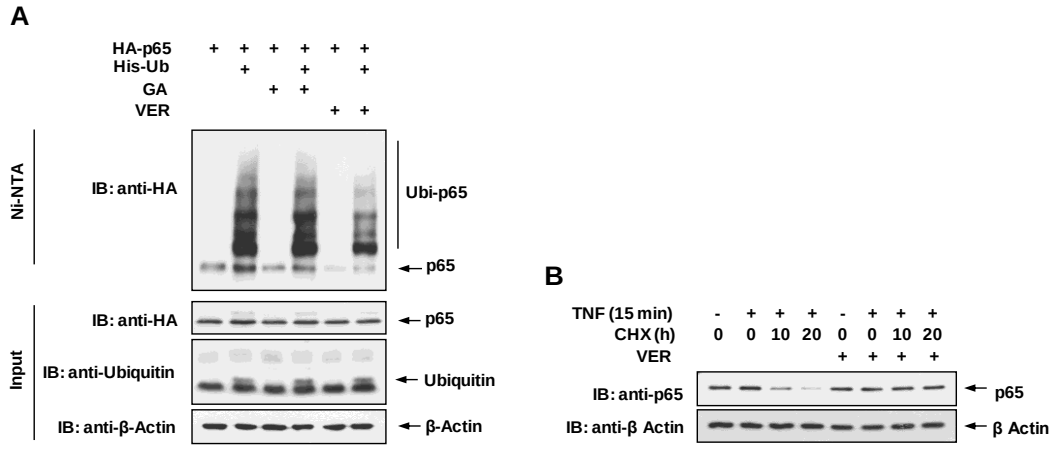



**Figure 3.18 The Hsc/Hsp70 system regulates p65 ubiquitination.** (A) HEK-293T cells were transiently transfected with HA-tagged wild type p65 alone or cotransfected to express His-Ub. Cells were treated with vehicle, GA or VER for 17 h. Cells were lysed under denaturing conditions and His-tagged proteins were enriched on Ni-NTA beads. Eluted proteins were analysed by immunoblotting using the anti-HA antibody. One aliquot was used to confirm expression of proteins by Western blotting with appropriate antibodies (Input). (B) HeLa cells were stimulated with a pulse of TNF (10 ng/ml) for 15 min to induce p65 degradation. The medium was changed and cells were treated with CHX (10 µg/ml) to block p65 resynthesis for indicated periods. VER (40 µM) or vehicle control were added 4 h before the TNF stimulation. The stability of p65 was analysed by Western blotting. Equal loading was confirmed by detection of β-Actin.

In summary, these experiments show that variety of PTMs acting on the NF-κB p65 protein can be modulated upon interference with the chaperone function of Hsc/Hsp70 proteins.

### 3.2.4.2 Hsps modulate p65-mediated gene expression

To test whether Hsps regulate the transcriptional activity of p65, reporter gene assays were performed. HEK-293T cells were transfected with a luciferase reporter gene controlled by 3 κB sites along with a p65 expression vector. The next day, cells were further grown in the absence or presence of Hsp inhibitors for 17 h and reporter gene activity was measured. The presence of GA only slightly diminished p65-triggered luciferase activity, while VER treatment significantly impaired p65-driven gene expression (Fig. 3.19A). Alternatively, luciferase gene expression was induced by Gal4-p65 fusion proteins that are independent of NF-κB DNA binding, as the contact with DNA is mediated by Gal4. Again, Gal4-p65 induced luciferase activity was significantly impaired in the presence of the Hsc/Hsp70 inhibitor, showing that their chaperone activity contributes to p65-mediated transcription (Fig. 3.19B).

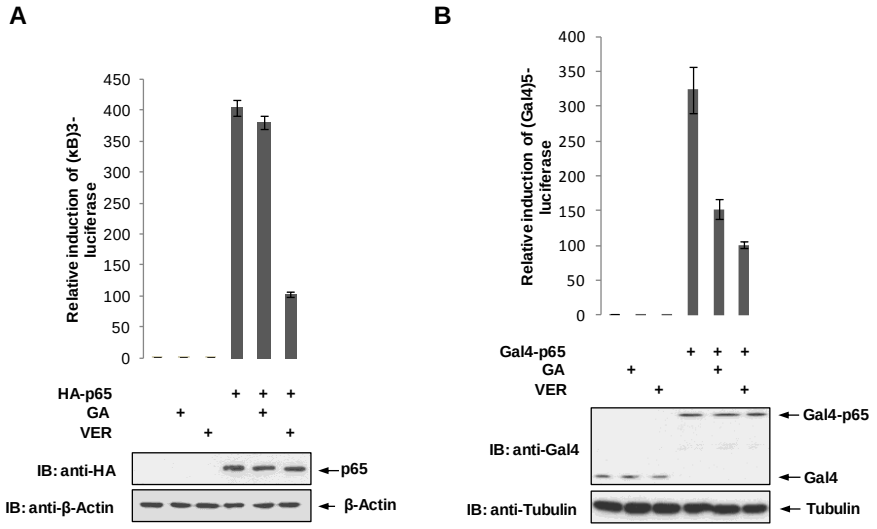



**Figure 3.19 Hsc/Hsp70 chaperones are important for p65-mediated transcription.** (A) HEK-293T cells were transfected with NF-κB-dependent luciferase reporter gene and an expression plasmid for HA-tagged wild type p65. Cells were treated with vehicle, GA (1 μM) or VER (40 μM) for one day, followed by cell lysis and determination of luciferase activity (upper panel) and protein expression (lower panel). Error bars show standard error of the mean (SEM) from three independent experiments. (B) HEK-293T cells were transfected with a luciferase reporter gene controlled by five Gal4-binding sites along with expression vectors for the DNA-binding domain of Gal4 fused to p65 (Gal-p65). Cells were treated with Hsp inhibitors and analysed for luciferase activity and protein expression as in (A). Error bars represent SEM from three independent experiments.

## 3.3 Influence of p65 DNA-binding ability on its conformation and activity

### 3.3.1 Characterization of stably reconstituted p65-/- MEFs

To reveal the functional importance of p65 DNA-binding for the TNF-induced p65 conformational rearrangement and transcriptional activity, two p65 DNA-binding mutants were generated. One mutant was created by changing the glutamic acid at position 39 (Glu39) to isoleucine residue (p65 E39I) [278]. This site was chosen since it facilitates a direct contact with the DNA and enables proper positioning of residues within the DNA-binding loop L1 [15]. Another p65 mutant was created where additionally to E39I a residue within the NLS sequence was changed. In this mutant the arginine within the NLS, at position 302, was changed to glutamic acid (p65 E39I/R302E). This second mutation impairs nuclear translocation of p65 [279]. p65-/- MEFs were reconstituted with lentiviral constructs to stably express HA- tagged wild type p65, p65 DNA-binding (p65 E39I) or the double DNA/NLS (p65 E39I/R302E) mutants. Comparable expression of mouse p65 proteins and other NF-κB regulatory proteins was assessed by immunoblotting (Fig. 3.20A). The expression of IκBα and precursor p100/p52 were not reduced in cells expressing the p65 DNA-binding or double p65 DNA/NLS mutant. Even though genes encoding for IκBε, Rel-B and c-Rel are also NF-κB-dependent genes [280-282], expression levels of those proteins in p65-/- MEFs reconstituted with an empty vector was comparable to MEFs expressing wild type p65 or its mutated versions. The DNA-binding ability of p65 mutants was also tested. Empty vector and the different HA-tagged p65 forms were expressed in HEK-293T cells. Whole cell extracts were prepared in TOTEX lysis buffer and tested for DNA-binding by EMSA experiment. The HA-tagged p65 E39I and p65 E39I/R302E mutants failed to bind to an oligo containing a canonical κB site (Fig. 3.20B). EMSAs combined with supershift assay confirmed the binding of wild type p65 and the loss of DNA-binding ability for p65 E39I and p65 E39I/R302E mutants (Fig. 3.20C).

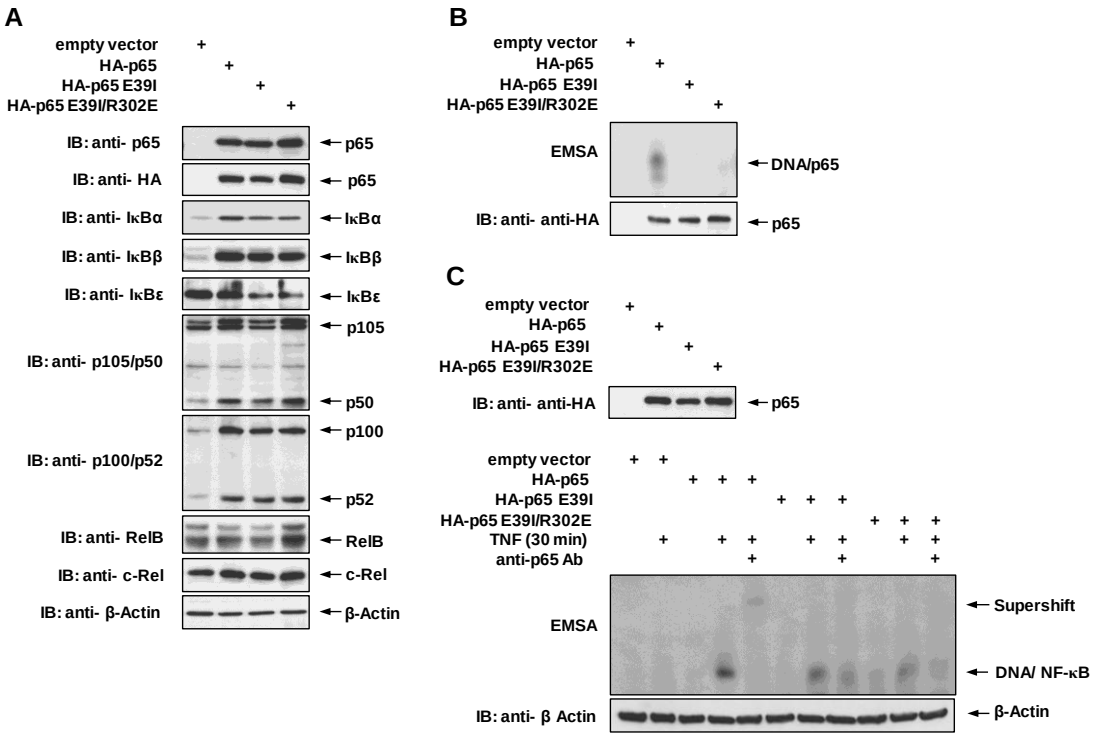
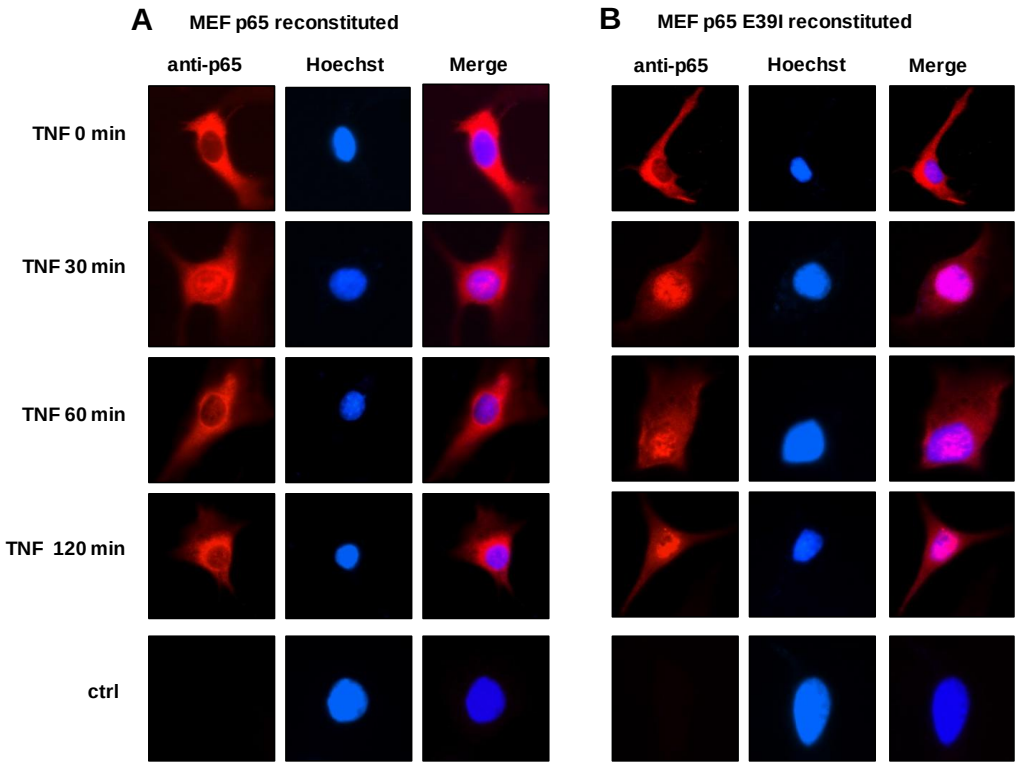



**Figure 3.20 Characterization of stably reconstituted p65-/- MEFs.** (A) The indicated murine HA-tagged wild type p65 and its mutated versions were stably expressed after lentiviral infection. Expression of p65 and other NF-κB regulatory proteins were assessed by immunoblotting as indicated. (B) HA-tagged wild type p65, p65 E39I or p65 E39I/R302E were expressed in HEK-293T cells. Whole cell extracts were prepared and tested by EMSA experiment for the ability to bind to a κB-oligonucleotide. Upper panel: an autoradiograph with the NF-κB/DNA binding complex, indicated by an arrow. Lower panel: an immunoblot with anti-HA antibody ensuring the correct expression of HA-tagged proteins. (C) p65-/- MEFs transduced with lentiviruses to stably express similar levels of HA-tagged p65 and its indicated mutated forms were left untreated or were treated with TNF (10 ng/ml) for 30 min. Total cell extracts were prepared. A p65-specific antibody used for supershift assay was added as indicated and the DNA binding ability of p65 versions was assayed by EMSA. After separation of free and NF-κB-bound oligos by native gel electrophoresis, the gel was dried and autoradiographed (lower panel). The upper panel represents a control Western blot ensuring that comparable amounts of protein were contained in the EMSA samples. Ab, antibody.

### 3.3.2 DNA-binding of p65 affects the kinetics of its nuclear export and Ser468-phosphorylation

Kinetics of NF-κB nuclear translocation was tested in stably reconstituted p65-/- MEFs. The reconstituted MEFs were stimulated with TNF for 0, 30, 60 or 120 min and the kinetics of the nuclear import and export was analysed by IF experiments (Fig. 3.21 A-C). In cells reconstituted with wild type p65, p65 was detected inside the nucleus after 30 min TNF stimulation and the nuclear export was visible after 60 min post-stimulation (Fig. 3.21A). Unexpectedly, TNF-activated p65 E39I mutants retained inside the nucleus even 120 min after stimulation (Fig. 3.21B). IF detected a slight nuclear translocation of the

double mutant p65 E39I/R302E upon TNF stimulation which probably occurs by a “piggy back” mechanism (Fig. 3.23C). Additionally, the kinetics of the TNF-induced p65 nuclear translocation and p65 phosphorylation was tested by a Western blot analysis. Reconstituted MEFs were stimulated with TNF for indicated periods. Immunoblotting of nuclear extracts revealed prolonged nuclear localization of p65 DNA-mutants in comparison to wild type p65. Upon TNF treatment, phosphorylation of wild type at Ser468 reaches its maximum at 15 min, followed by a gradual decrease of the Ser468-phosphorylation signal. Interestingly, in cells expressing the p65 DNA-binding mutant, Ser468 phosphorylation reached its maximum at 15 min TNF stimulation and did not change during the following time course of stimulation (Fig. 3.21D). Kinetics of Ser563 phosphorylation remained unchanged. All together, results presented in Fig. 3.21 imply that the p65 DNA-binding is involved in regulation of the p65 nuclear export and affects phosphorylation at Ser468.



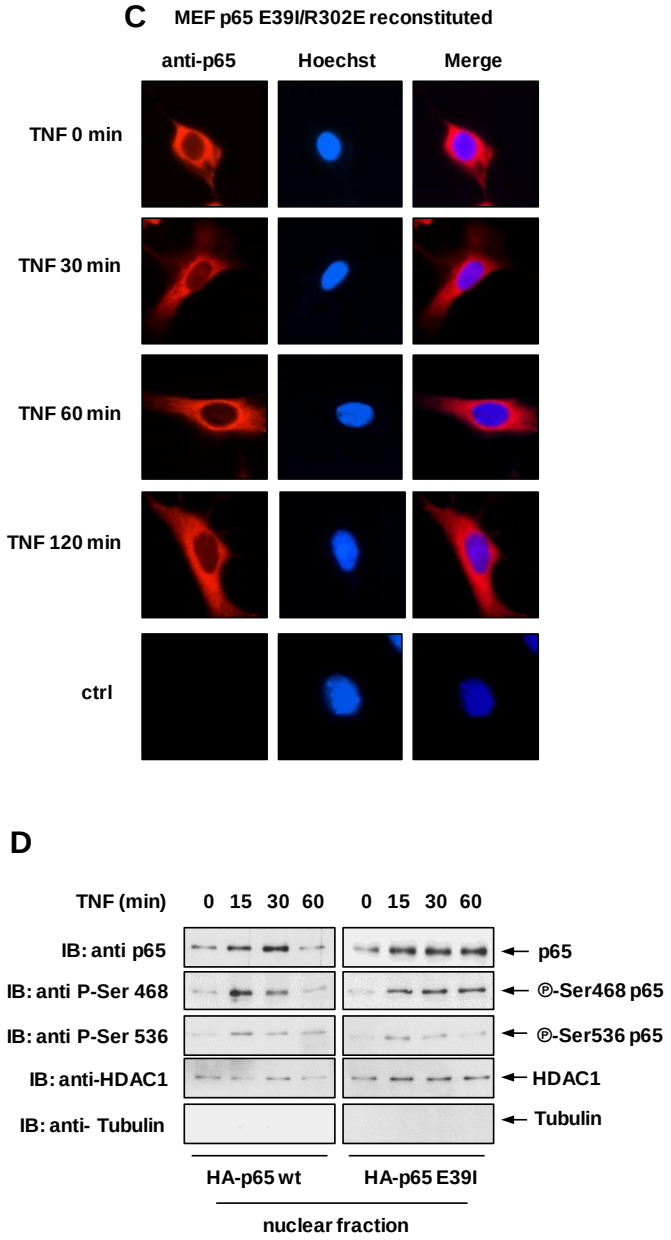



**Figure 3.21 Kinetics of NF-κB nuclear translocation in stably reconstituted p65-/- MEFs.** (A-C) p65-/- MEFs were stably reconstituted to express either murine HA-tagged wild type p65 (A), DNA-binding mutant p65 E39I (B) or double mutant p65 E39I/R302E (C) were stimulated with TNF (10 ng/ml) for indicated periods. Cells were fixed and stained at different time points with an antibody against p65. Chromosomal DNA was stained with Hoechst and merged images indicate cellular localization of p65. (D) Stably reconstituted MEFs were stimulated with TNF (10 ng/ml) for indicated periods. Nuclear extracts were prepared and analysed by SDS-PAGE and immunoblotting with indicated antibodies.

### 3.3.3 The impact of p65 DNA-binding on TNF-induced p65 conformation

It has been suggested that the composition of NF-κB binding sites affects the configuration of a bound NF-κB dimer that further dictates the interaction with specific coactivators [259]. As previous results from this study showed that cytokine-induction triggers a change in p65 conformation, the relevance of p65 DNA-binding on TNF-induced conformational change was investigated. Reconstituted MEFs with HA-tagged wild type p65 or p65 E39I were left unstimulated or stimulated with TNF for 15 min after which the nuclear extracts were subjected to limited proteolysis experiments with trypsin. Immunoblotting with a cocktail of p65-specific antibodies did not reveal any significant difference in the digestion

patterns of wild type p65 and the DNA-binding mutant (Fig. 3.22A). To reveal the contribution of DNA-binding by an alternative experimental approach, nuclear extracts from TNF-stimulated MEFs were incubated with or without a DNA oligo containing the consensus κB site to allow p65 DNA-binding in vitro. Limited proteolysis assays failed to detect changes in the digestion pattern (Fig. 3.22B, lane 2). Collectively, these results show that the TNF-induced conformational change of p65 occurs independently of its binding to DNA.

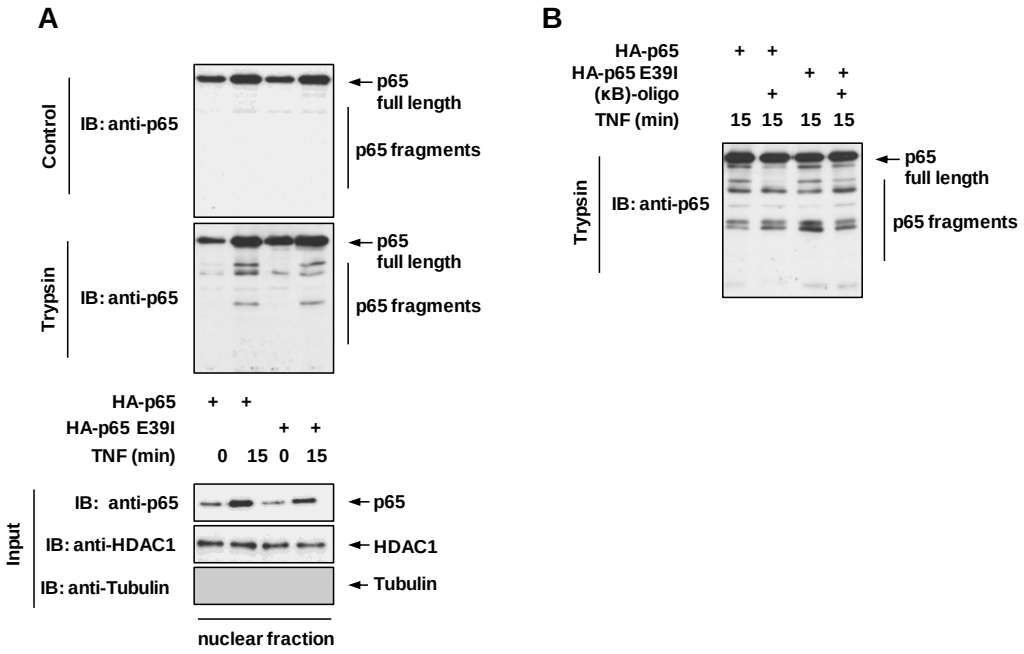



**Figure 3.22 The TNF-induced conformational change is DNA-binding independent.** (A) p65-/- MEFs were transduced with lentiviral vectors to stably express similar levels of HA-tagged p65 and its DNA-binding mutant version HA-p65 E39I. Cells were stimulated with a pulse of TNF (10 ng/ml) for 15 min. Nuclear extracts were prepared and 10% were used for an input control to ensure correct expression of HA-tagged proteins, purity of the nuclear fraction and equal sample loading. The rest of the lysate was digested with limited amounts of trypsin. p65 fragments were separated by SDS-PAGE and digestion patterns were detected by immunoblotting with anti-HA antibody. (B) Aliquots of the same nuclear extracts from TNF-treated reconstituted MEFs used in (A) were incubated alone or with an oligonucleotide harboring a NF-κB binding site. After incubation for 60 min on ice and digestion with limited amounts of trypsin, the fragmentation of p65 was detected with p65-specific antibodies.

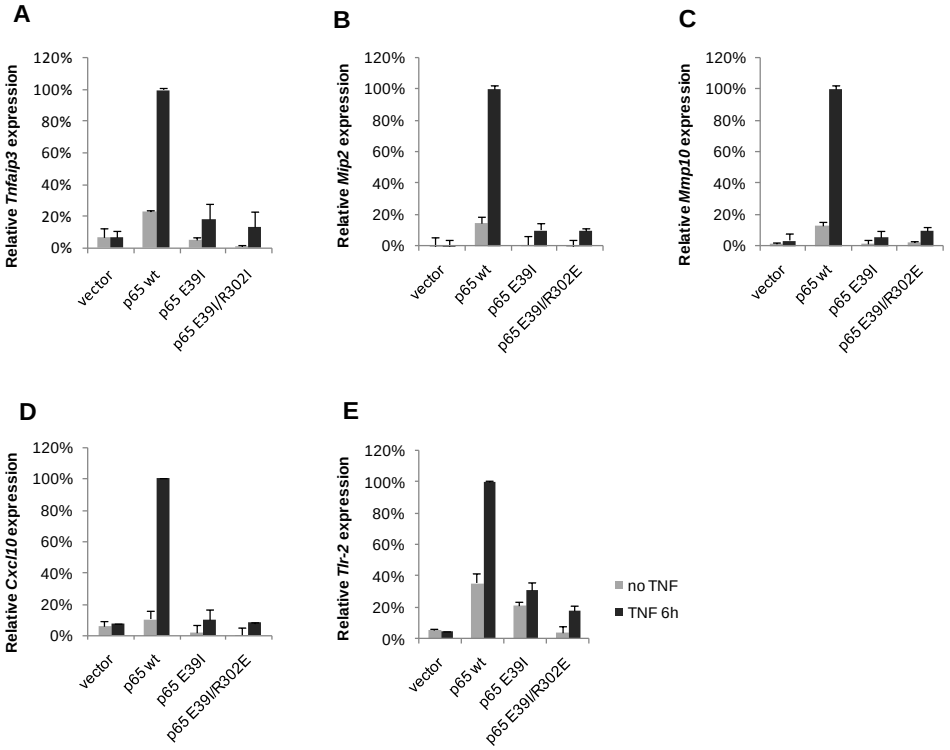
### 3.3.4 Expression of some TNF-induced p65 dependent genes is not dependent on p65 DNA-binding

In order to induce transcription of its target genes, NF-κB p65 binds to DNA and interacts with various TFs, coactivators or components of the basal transcription complex [283]. To analyse the importance of p65 DNA-binding in gene expression, stably reconstituted p65-/-

MEFs were left untreated or stimulated with TNF for 6 h and expression of several known p65-target genes (Table 3.1) was quantified by qRT-PCR (Fig. 3.23 A-K). A set of chosen genes including *Tnfaip3, Mip2, Mmp10, Cxcl10, Tlr2* were induced only in cells reconstituted with wild type p65 (Fig. 3.23A-E). Interestingly, genes such as *Il-6, Ccl9, JunB, Saa3, Ptgs2* and *Nfκbiα* were not depending on p65 DNA-binding (Fig. 3.23F-K), suggesting that p65 is recruited to its target sites by protein-protein interactions.

**Table 3.1**

| Gene | Function | Reference |
|---|---|---|
| *Ccl9* | Chemokine | [201] |
| *Il-6* | Inflammatory cytokine | [201] |
| *Cxcl10 (Ip10)* | Chemokine | [284] |
| *Mip-2* | Macrophage inflammtory protein-2, chemokine | [182, 284] |
| *Tlr-2* | Toll-like receptor | [285, 286] |
| *Saa3* | Serum component | [166] |
| *Ptgs2 (Cox-2)* | Cyclooxigenase | [287, 288] |
| *Tnfaip3(A20)* | TNF-inducible zinc finger | [201, 288] |
| *Nfkiba (IκBα)* | Inhibitor of *Rel*/NF-κB | [201, 288] |
| *Junb* | Proto-oncogene | [201, 288] |
| *Mmp10* | Secreted colagenase involved in metastasis | [201] |



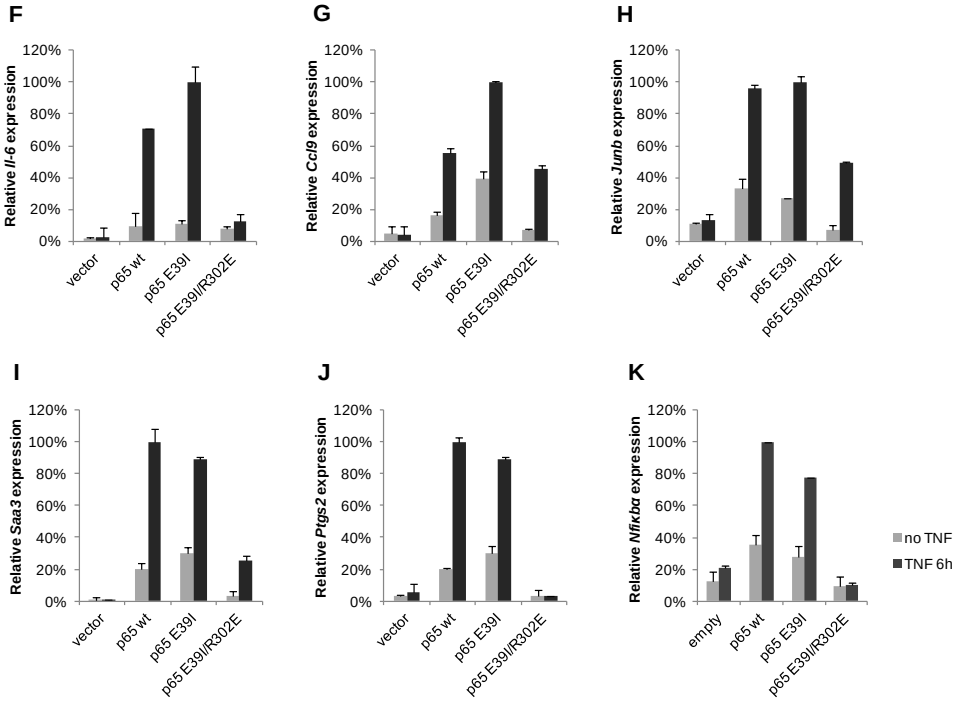



**Figure 3.23 Identification of genes which are dependent or independent on p65 DNA-binding.** p65-/- MEFs reconstituted to stably express similar levels of HA-tagged versions of p65 were stimulated with TNF (10 ng/ml) for 6 h or left untreated. Gene expression was determined by qRT-PCR and normalized to the level of β-Actin. TNF-inducible target genes were grouped into p65 DNA-binding dependent (A-E) and independent (F-K) genes. In order to facilitate comparison, maximal gene activation was arbitrarily set as 100%. Experiments were performed in triplicates, error bars display standard deviations.

# 4. Discussion

## 4.1 Phosphorylation is important for the conformation of the active nuclear p65

The NF-κB transcription factor is involved in the control of a large number of cellular processes, such as immune and inflammatory responses, developmental processes, cell growth and apoptosis [1, 289]. The cellular localization, activity and termination of NF-κB activity must be carefully controlled in response to intrinsic and environmental stimuli. Many aspects of these processes rely on changes in the folding state of the transcriptionally highly active NF-κB p65 subunit. Inside the nucleus, the function of NF-κB is greatly regulated by various stimulus-induced PTMs of the p65 subunit. Early studies on p65 showed that the TAD of p65 is binding its own RHD through intramolecular associations [154, 290]. Stable physical interaction between the p65 N- and C-termini was detected by GST pull-down assays. Protein kinase A-mediated phosphorylation of Ser276 abrogated this interaction, implicating the role of phosphorylation in the regulation of p65 conformation [154]. A previous study from the Schmitz lab showed that mutation of a single phosphorylation site in p65 (Ser468) largely prevents the association with components of a multimeric ubiquitin ligase complex and subsequent p65 ubiquitination, suggesting that this mutation alters the overall conformation of NF-κB p65 [182]. In an effort to measure the conformational changes of p65 mediated by site-specific phosphorylation, limited proteolysis assays were performed using cells expressing either the wild type p65 or individual p65 Ser276, Ser468 and Ser536 mutated forms. Interestingly, proteolysis resulted in a different fragmentation of each p65 version (Fig. 3.2B). These results suggest that the phosphorylation status of individual serine residues affects the overall conformation of p65, indicating a highly dynamic behavior of the protein. Since NF-κB p65 undergoes simultaneous phosphorylation at different sites upon stimulation, these results raise the possibility that p65 can adopt more than one conformation. It remains to be elucidated whether there are only two or even more different conformations. It has been suggested that phosphorylation at Ser276 indirectly regulates the DNA-binding affinity of p65 [24] and its dimerisation with other NF-κB monomers [4]. Also, site-specific phosphorylation of p65 regulates p65 affinity to

modifying enzymes and/or transcriptional cofactors [154, 164, 165, 183, 291]. Thereby, in agreement with the proteolysis assays, all these results could be explained by a highly dynamic flexibility of p65 that allows p65 to respond under diverse conditions. The intrinsic disorder of the C-terminal portion also contributes to the dynamic behavior of the p65 protein (Fig. 3.1).

Conformational changes induced by a site-specific or multiple-site phosphorylations were detected for various TFs such as p53 [263], the glucocorticoid receptor (GR) [292], signal transducer and activator of transcription 1 (STAT1) [293, 294], FoxO1 (Forkhead box O1) [295] and Elk-1 (ETS domain-containing protein1) [296]. For example, a certain phosphorylation of FoxO1 within its DBD induces a conformational change that blocks FoxO1 DNA-binding [295]. Phosphorylation of the Elk-1 C-terminal TAD induces a change in the intramolecular interaction between the C-terminal TAD and the N-terminal DBD that accompanies the stimulation of DNA binding [296]. The exact contact sites between the N- and C-terminal halves of p65 remain to be identified. As the intrinsically disordered C-terminal half of the p65 protein does not allow structural data for the entire p65 protein [32], biochemical experiments with purified parts of p65 could determine contact sites or protein regions that are responsible for intramolecular interaction.

Limited proteolysis experiments with comparable amounts of TNF or IL-1-induced p65 showed no significant cytokine-inducible changes for cytosolic p65, while nuclear p65 displayed a major conformational rearrangement (Fig. 3.5A and 3.6A). Treatment with the phosphatase inhibitor calyculin A in the absence of cytokine stimulation was sufficient to cause a p65 rearrangement highly similar to cytokine-induced refolding, suggesting that phosphorylations are major contributors of p65 rearrangement (Fig. 3.7A). The limited proteolysis experiments with p65-/- MEFs stably reconstituted with non-phosphorylatable Ser468 or Ser536 p65 mutants did not show a significant effect of those two phosphosites on p65 conformation (Fig. 3.7B). This indicates that phosphorylation at Ser 468 or 536 alone is not sufficient to induce a conformational change, but other modifications need to occur as well. Besides phosphorylation, other induced PTMs such as acetylation [297] methylation [298], ubiquitination [299, 300], sumoylation [301] and prolyl-isomerisation [164, 263] can affect protein conformation. The phosphorylation site(s) responsible for a cytokine-induced conformational switch needs to be revealed in the future by identifying p65 mutants refractory to the cytokine-induced refolding. As TNF and IL-1 induce a

variety of different PTMs [164, 170, 182, 214, 302, 303], their contribution to refolding of nuclear p65 should be investigated. It is presently also completely unclear whether the other NF-κB activating stimuli such as LPS, UV light and genotoxic stress induce the same conformational switch or possibly allow alternative configurations. It has been shown that TNF stimulation induces the COMMD1-mediated p65 ubiquitination through Ser468 phosphorylation [182], while the aspirin-induced COMMD1-mediated p65 ubiquitination occurs independently of Ser468-phosphorylation [184]. Cell stimulations with other NF-κB activating stimuli followed by a limited proteolysis should be performed in order to reveal whether a configuration of an active nuclear p65 is stimulus-specific.

In this study limited proteolysis assays were used to reveal conformational features of p65. Unlike other methods it allows the analysis of protein conformations using low protein concentrations. Protein regions with a high structural flexibility, such as loops and hinges, are usually most susceptible to proteolytic cleavage [304, 305]. As many phosphorylation, methylation, acetylation and ubiquitination sites predominantly locate in flexible, disordered regions [306-308], the outcome of a limited proteolytic protein can be influenced by PTMs. Of note, the modification sites mutated in this study did not change the predicted cleavage by trypsin or thermolysin. Also protein-protein interactions could affect protease susceptibility by masking the cleavage sites. The kinetics of a TNF- or IL-1-induced p65 refolding showed that characteristic conformational change of nuclear p65 is not transient, at least for the time points that were measured (Fig. 3.6B). It still can be detected at time points when the first wave of NF-κB activity is terminated by newly synthesized IκBα [69]. Thus, it would be interesting to investigate whether the changed digestion pattern disappears at later time points. To investigate the role of IκBα in p65 refolding in more detail, the kinetics of cytokine-induced p65 refolding should be measured in IκBα-/- cells or alternatively in cells with silenced IκBα. A previous study showed that cytokine stimulation induces the phosphorylation at Thr254 and leads to the binding of the prolyl isomerase Pin-1 to the phospho-Thr254/Pro motif in p65 [164]. Ryo *et al.* further showed that the subsequent phosphorylation-dependent isomerisation of p65 by Pin1 inhibits p65 binding to IκBα and results in increased nuclear accumulation and protein stability of p65. Thereby the detected rearrangement of p65 could be influenced by interaction with Pin1. The treatment of cells with a Pin1 inhibitor did not show any effect on TNF-induced p65 rearrangement (data not shown). Alternatively, the kinetics of a

cytokine-induced p65 refolding could be performed in Pin1-deficient cells. Further identification of digested fragments, especially p65* double band, by MS would give information about the trypsin cleavage sites of p65 which become accessible after refolding. Also, this could identify functional subregions within p65.

Immunoprecipitation of nuclear extracts from TNF stimulated cells with a set of p65-specific antibodies allowed the identification of a conformation-specific p65 antibody (EP2161Y) (Fig. 3.8B). Interestingly, cytokine stimulation leads to the exposure of a Ser468 surrounding region, emphasizing the regulatory role of the TAD2 subdomain containing Ser468 as a dynamic region within nuclear p65. It would be desirable to generate more conformation-specific antibodies, as they were successfully developed for the p53 protein [309]. Identification of antibodies that recognize constitutively exposed epitopes (e.g. IMG-5915A) and the epitope that become hidden after the TNF-stimulation would contribute to a better characterization of the observed cytokine-induced rearrangement (Fig. 4.1). The conformation-specific p53 antibody PAb240 that recognizes the mutant-specific epitope was able to immunoprecipitate p53 bound to Hsp70 or MDM2 (Mouse double minute 2 homolog), while the antibodies that were able to recognize the wild type-specific epitope did not [241, 300]. Thereby, conformation-specific p65 antibodies could be further used for the identification and characterization of conformation-specific p65 protein-protein interactions as well as interactions of NF-κB with chromatin and DNA.

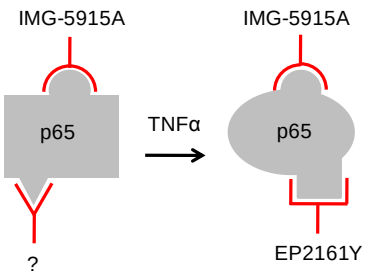



**Figure 4.1 Conformation-specific antibodies to monitor cytokine-induced rearrangement of p65.** IMG-5915A recognizes a constitutively exposed epitope, EP2161Y preferentially recognizes TNF-inducible exposed epitope. An hypothetical antibody (?) recognizing the epitope that would be concealed after the TNF stimulation.

The detailed role of site-specific PTMs (e.g. phosphorylation) in the function of individual proteins remains poorly understood. Structural information is particularly limited, as it is difficult to obtain sufficient amounts of purified protein in a specific modification state. Several methods could be further employed in order to examine protein structural changes induced by phosphorylation. Atomic-resolution structures for the phosphorylated proteins can be determined by X-ray crystallography. For phosphorylated peptides and small

peptides in solution NMR is the method of choice. Several NMR studies of phosphorylation-induced conformational changes have been reported [262, 292, 310-313]. Electron paramagnetic resonance (EPR) and circular dichroism (CD) experiments can also provide information about the conformational change due to the phosphorylation, but do not provide atomic details. Computational modeling studies can be helpful to enlighten the principles and mechanisms of PTMs [262]. Size exclusion chromatography in combination with analytical ultracentrifugation can be also applied to characterize hydrodynamic and conformational changes in proteins [314, 315]. Hydrogen exchange coupled to MS has become a valuable analytical tool for the study of protein dynamics and can provide information on the protein structure [316].

## 4.2 Phosphorylation-induced p65 refolding regulates p65 ubiquitination

The phosphorylation-dependent dynamic behavior of p65 could serve to explain the differences in the level and type of ubiquitination of wild type p65 and its phospho-mutants (Fig. 3.3 and 3.4). Mutation of a single phosphorylation site in the disordered C-terminal TAD resulted in a strongly decreased p65 ubiquitination in comparison to wild type p65. These results suggest that a serine to alanine mutation of C-terminal residues could induce an intramolecular rearrangement that affects the exposure of ubiquitination sites. On the other hand, it cannot be excluded that phosphorylation at Ser468 and Ser536 induces exposure of docking motifs through which the interaction with the Cul2/COMMD1 complex occurs, facilitating the SOCS1-p65 interaction [182, 183, 210]. Also, it can be possible that the binding of a Cul2/COMMD1 complex itself, induces the change in p65 conformation. A phosphorylation-dependent control of ubiquitination was also observed for the p53 protein. Binding of the E3 ligase MDM2 to the intrinsically disordered p53 N-terminus induces a conformational change in wild type p53, as revealed by the p53 immunoprecipitation with a mutant conformation-specific p53 antibody [300]. Phosphorylation of N-terminal Ser20 contributes to disruption of p53-MDM2 interaction, especially in combination with Ser15 and Thr18 phosphorylation [317-319]. Still, mutation of Ser15 to alanine had little effect on the ubiquitination and degradation of p53. In contrast, replacement of Ser20 with alanine makes p53 highly sensitive to MDM2-mediated turnover [320]. The cross-talk between the p65 C-terminal phosphorylation and ubiquitination requires further detailed investigation. Serine to alanine mutation of Ser276 led to the p65 ubiquitination, while the permanent phosphorylation inhibited p65

ubiquitination. In agreement with a Ser276 phosphorylation-induced p65 refolding, it is possible that mutation to alanine makes ubiquitin acceptor sites available for modification. This result is in agreement with a previous study where it has been shown that Pim-1-mediated phosphorylation at Ser276 prevents SOCS1-dependent ubiquitination and subsequent degradation [321]. Lysines are also subjected to acetylation and methylation [200, 201, 214, 218]. The observed effect of phosphorylation at Ser276 on p65 ubiquitination could be a consequence of a p65 interaction with acetyl- or methyltransferase. It is well documented that Ser276-phosphorylation stabilizes the interaction with the CBP/p300 [154, 322]. This interaction could induce further lysine modifications and/or protects the other ubiquitin acceptor lysines, leading to changes of p65 conformation. The highly dynamic p65 flexibility would also help to explain previous experiments that characterized a p65 variant mutated in four out of seven ubiquitinated lysines. While the exchange of four lysines largely prevented p65 ubiquitination, mutation of further lysines paradoxically further increased p65 ubiquitination [201], a finding that may reflect large conformational changes leading to the surface exposure of additional lysines.

## 4.3 Phosphorylation-induced conformational changes of p65 influence its association with other proteins

Coimmunoprecipitation experiments showed that a conformational change induced by Ser468 phosphorylation elicits significant effects on p65 protein-protein interactions (Fig. 3.9 and 3.12). This supports the finding that Ser486 and its surrounding sequence in the TAD2 subdomain are of special relevance for a configuration of active nuclear p65. Phosphorylation-induced conformational changes in proteins are largely driven by electrostatic perturbations that change the protein backbone structure of a surrounding region [262] and induce the exposure of otherwise buried enzyme docking sites [323]. The region surrounding the Ser468 (Ser467 for murine p65) contains two ΨXXΨΨ motifs (Ψ-hydrophobic residue, X-any residue; $^{449}$LGALL$^{453}$ and $^{473}$FQQLL$^{477}$, respectively for murine p65) which are a generalization of a LXXLL motif that is known to mediate protein-protein interactions in transcriptional regulation [324]. Recently it has been shown that hydrophobic residues from those motifs create a platform for the interaction with the CBP/p300 TAZ1 (transcriptional adaptor zinc binding 1) domain allowing the disorder-to-order transition of the unstructured p65 TAD2 subdomain [180]. Isothermal calorimetric

(ITC) experiments with p65 TAD2 fragments showed that a phospho-mimetic p65 S467D fragment binds TAZ1 with a slightly higher affinity than wild type p65 TAD2, suggesting that the increased negative charge of phosphoserine is not essential for the interaction but would enhance CBP TAZ1 binding even further over nonphosphorylated p65. GST pull-down assay revealed that the TAD1 subdomain of p65 provides the main region for binding of the cofactor GCN5 [183]. Still, the individual or combined mutation of all six serines in TAD1, including the Ser536, did not abrogate the inducible binding between p65 TAD and GCN5. Interestingly, the mutation of Ser468 to alanine affected the interaction between p65 and GCN5 in response to either calyculin A or TNF treatment, suggesting that phosphorylation of p65 at Ser468 increases its binding to GCN5 [183]. Another LXXLL-like motif ($^{542}$FSALL$^{546}$, respectively for human p65) is positioned close to the Ser536. Phosphorylation of Ser536 also has the ability to control protein association, as exemplified by the increased association of the transcriptional regulator TAFII31 [170]. Future experiments could investigate in more detail the role of phosphorylations on the exposure of the FXXLL motifs. Also, the impact of ψXXψψ motifs on the interaction with transcriptional coregulators in general could be tested. Phosphorylation-induced conformational changes regulating the interaction with transcriptional cofactors have been also detected for other TFs [292, 325, 326]. Limited proteolysis assays together with CD and fluorescence emission spectroscopy showed that single-site phosphorylation in intrinsically disordered N-terminal TAD of the GR is sufficient to induce a functionally active conformation of GR [292]. This phosphorylation-induced rearrangement of GR further facilitates interaction with transcriptional coregulators and subsequently GR transcriptional activity.

This study showed that p65 interaction with Hsp70 and Hsp90 was also influenced by phosphorylation of Ser468 (Fig. 3.12). Exposed stretches of hydrophobic residues were also identified as regions through which Hsp70 binds to its substrates [327] and the conformation of substrate peptides plays a pivotal role in the binding process [328]. Further experiments could be performed with the aim to investigate whether the p65 TAD ψXXψψ motifs participate in NF-κB p65-Hsp70 interaction. A recent study performed by Neil Perkins and his colleagues showed that in the absence of p53, p65 was transported into the mitochondria and recruited to the mitochondrial genome by an interaction between the Thr505 region of the p65 TAD and Mortalin, a mitochondrial Hsp70 protein [258].

Mutation of Thr505 to alanine abolished the p65 interaction with Mortalin and inhibited p65 mitochondrial localization, suggesting that phosphorylation at this site may be required to promote this interaction. The authors do not exclude the possibility that this can also happen due to the structural effect of a serine to alanine mutation. While the data presented here did not reveal the importance of Ser536 phosphorylation on the interaction of p65 with Hsp70 (data not shown), still the impact of Thr505 phosphorylation on the p65/Hsp70 association could be tested in the future. Phosphorylation-dependent binding of Hsp70 to its substrate was detected for protein kinase C (PKC), Akt/protein kinase B and PKA [243]. GST pull-down assays with wild type PKC or a kinase variant lacking phosphorylated amino acids in a C-terminal turn motif revealed that Hsp70 binds to the unphosphorylated PKC turn motif. This interaction of Hsp70 with PKC allows the re-phosphorylation of the enzyme and sustains the activated state of protein kinase. All together, these examples readily illustrate that phosphorylation-mediated conformational changes of p65 can greatly extend and specify the repertoire of possible protein-protein interactions. Methods like surface plasmon resonance (SPR) and ITC can be employed on a series of truncated and mutated purified parts of a p65 to measure the p65 protein-protein interactions and to understand what regions of the interface are most important for the tight binding affinity of protein complexes. GST pull-down assays with purified Hsps and truncated p65 fragments should be performed in order to see whether they directly interact with p65 or via co-chaperones.

## 4.4 Chaperones as coregulators of p65 refolding

Hsp70 and Hsp90 are highly abundant cellular proteins that play a dominant role in the cellular system that controls conformational states and conformational maturation of proteins [329, 330]. In this study the pharmacological inhibition of Hsc/Hsp70 and Hsp90 revealed that chaperones have no impact on folding of newly synthesized p65 (Fig. 3.13) and maintenance of the p65 conformation under basal conditions (Fig. 3.14). In the presence of the Hsc/Hsp70 inhibitor VER TNF-induced refolding of p65 occurred, but the overall digestion pattern was slightly changed (Fig. 3.15). This suggests that chaperone activity of Hsc/Hsp70 is not crucial for the p65 refolding *per se*, but rather for the fidelity of TNF-induced conformational rearrangements. The absent effect of Hsp90 inhibition on the p65 conformational change could be a consequence of weak interactions between p65 and Hsp90. Alternatively, Hsp90 could help in the folding of p65 interaction partners.

Many intrinsically disordered (ID) TFs including p53, HSF-1 (heat shock transcription factor-1) and hormone receptors interact with Hsp70 and Hsp90 [231, 242, 257, 329, 331, 332]. At physiological temperatures Hsp90 alone stabilizes wild type p53 in a folded, active state and influences the DNA-binding and transcriptional activity of p53 in vivo [333, 334]. Under stress conditions such as heat-shock, the chaperones Hsp90 and Hsp70 maintain the native conformation of p53. It has been shown that the DBD of wild type p53 becomes rather loose and less structured during its interaction with Hsp90 [329] and that ATP-dependent dissociation of the p53-Hsp90 complex allows further folding of the p53 protein to an active conformation which is capable of DNA-binding [335]. As p65 and Hsp70 constitutively interact, the details of the p65-Hsc/Hsp70 complex should be revealed. In particular it would be interesting to investigate the impact of this interaction on the p65 TAD flexibility under basic conditions and after stimulation. As the p53 protein mainly interacts with Hsp70 and Hsp90 via its structured DBD [334, 336], future experiments could test whether also p65 uses its DBD for the interaction with Hsps. The relative contribution of phosphorylations and chaperones for refolding of p65 is schematically shown in Figure 4.2.

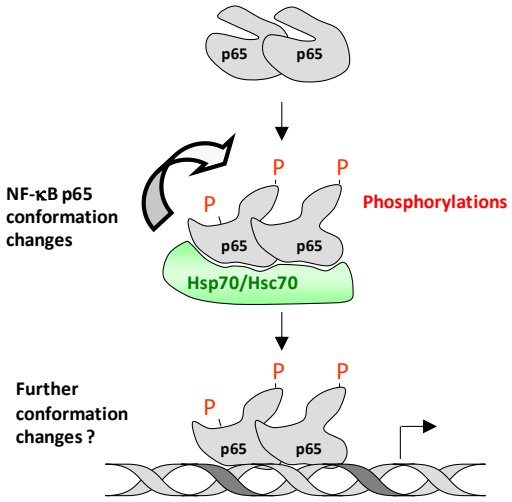



**Figure 4.2 Schematic model displaying an induced conformational change of p65.** The NF-κB subunit p65 becomes phosphorylated which leads to the change in the p65 conformation. Constitutively associated Hsc70/Hsp70 proteins control the fidelity of p65 refolding.

Some ID proteins (IDPs) like tau [337], amyloid fibril [338] or α-synuclein require the involvement of chaperones for function. Still, a study based on protein interaction databases showed that IDPs do not show preferential binding to chaperones [339]. The presence of structural disorder negatively correlates with the tendency of IDPs to be chaperones binding partners and the authors suggest that IDPs does not need chaperone assistance for folding, but probably for assembly into protein complexes and protection

from aggregation. Furthermore, it has been detected that chaperones could promote degradation of several IDPs, including p53, Tau, and HIF-1 [340]. In the present study VER treatment inhibited TNF-induced p65 degradation, thus it will be interesting in future experiments to investigate the role of Hsp70 on p65 stability (Fig. 3.18B).

Hsp70 functions in cooperation with co-chaperones (e.g. Hsp40) and various NEFs where these proteins assist protein folding during translation or post-translationally. The recently proposed N-terminal folding nucleation hypothesis suggests that the generation of intrinsically folded N-terminal regions at the ribosome serve as nucleation points for the yet unstructured C-terminal portions of the peptide chains [341]. This mechanism is likely to regulate the folding of a newly synthesized p65 protein. According to this hypothesis, a structured p65 N-terminal half would allow for specific intramolecular protein contacts with the unstructured C-terminal half. This would generate a compact protein shape which allows prevention of protein aggregation and proteolysis. On the other hand, if a protein has a disordered N-terminus, its degradation can be potentially initiated when it starts to emerge from the ribosomal channel. These proteins usually require chaperones for folding. For example, p53 has an unstructured N-terminal domain and a recent study showed that its N-terminus binds the chaperonine CCT (chaperonin-containing t-complex polypeptide 1 (TCP1)) which plays an important role in the folding of newly synthesized proteins [342-344]. The identification of intramolecular p65 contact sites would help to investigate the role of the N-terminal hypothesis in p65 folding.

## 4.5 Chaperones as coregulators of p65 PTMs and p65-mediated gene expression

Treatment with Hsp inhibitors led to changed p65 PTMs. This effect can be due to several reasons including effects on Hsp-assisted refolding of p65. On the other hand, Hsps could also be important for proper conformation of the p65 modifiers themselves. It has been shown that GA treatment reduces IKKα and IKKβ kinase activities, which in turn resulted in decreased IκBα phosphorylation [248, 249, 255]. The loss of Ser536 phosphorylation in the presence of GA can therefore be explained by impaired IKK complex kinase activity (Fig. 3.16). The reason for diminished Ser468 phosphorylation in the presence of GA could be due to impaired activity of the kinase(s) or phosphatases regulating the phosphorylation state of this residue. As IKKε is important for Ser468 phosphorylation [179], further

experiments should be performed to reveal whether inhibition of the Hsp90 system affects IKKε activity. It is reasonable to assume that other p65 phosphorylation sites are affected upon inhibition of the Hsp90 system as well. The Pim-1 kinase phosphorylates p65 at Ser276 and is known to interact with Hsp90 and Hsp70 [345], thus Pim-1-mediated phosphorylation will depend on proper protein folding.

Inhibition of Hsp70 enhanced p65 Ser468 phosphorylation (Fig. 3.16). The kinase GSK-3β phosphorylates p65 at Ser468 under basal conditions [178], but it is also implicated in TNF-induced NF-κB activation [346]. GSK-3β is inactivated through Akt-mediated phosphorylation [347], while the dephosphorylation by the phosphatase PP1 converts GSK-3β into its active form [348]. As Hsp70 regulates the activity of the Akt kinase [243], inhibition of the Hsp70 ATPase activity could causes impaired Akt kinase activity. Loss of Akt-mediated phosphorylation-dependent GSK-3β inactivation would lead to permanent GSK-3β activity and enhanced p65 Ser468 phosphorylation. On the other hand, a higher p65 phosphorylation at Ser468 and Ser536 in the presence of VER treatment could be a consequence of impaired Hsp70/Hsc70-mediated regulation of serine/threonine phosphatases. One of the Hsp70 client proteins is the phosphatase slingshot, which regulates the phosphorylation-induced activity of the actin-binding protein cofilin [349]. Reduced p65 acetylation and ubiquitination in the presence of Hsp inhibitors could be a consequence of impaired p65 phosphorylations which can further affect p65 binding affinities to acetyltransferases, HDACs [154, 174, 180] or assembly with the COMMD1-E3 ligase complex [182, 183]. The role of the Hsc/Hsp70 system in regulation of p65 phosphorylation/dephosphorylation cycles and associated p65 refolding should be investigated in more detail in the future.

Decreased p65 acetylation caused by the Hsp inhibition can be a consequence of a direct effect of chaperones on acetyltansferase activity. Recently it has been shown that the chaperonin containing TCP1 subunit eta (CCTη) regulates p65 acetylation and NF-κB transcriptional activity by controlling CBP activity. CBP activity is regulated by auto-acetylation which contributes to its enzymatic activity [350]. Knockdown of CCTη reduced CBP acetylation and thus its activity. In CCTη knockdown cells the level of p65 acetylation was also reduced. The role of Hsp90 in regulation of GR-mediated gene transcription was also detected. Treatment with GA diminished GR-mediated gene transcription by blocking the GR and CBP/p300 recruitment to promoters [351].

Reporter gene experiments showed that chaperone activity provides an important contribution to p65-induced transcription (Fig. 3.19). The p65-triggered gene activity was only slightly diminished in the presence of GA, while VER significantly impaired p65-driven gene expression. These results are in agreement with a recent study where siRNA-mediated knock-down of Hsc70 was sufficient for strongly reduced NF-κB transcriptional activity [242]. Klenke *et al.* showed that interaction between Hsc70 and p65 is dependent on Hsc70 ATPase activity, since p65 interaction with Hsc70 mutant carrying a point mutation in the ATPase domain resulted in a loss of interaction. Chaperone inhibitors can affect the outcome of p65-dependent transactivation by preventing the proper folding of accessory proteins such as RNA Pol II and its numerous subunits and coregulators [352]. It was recently shown that Hsp90 binds the transcription start site (TSS) and maintains and optimizes RNA pol II pausing via stabilization of the negative elongation factor complex over the gene body [353]. Hsp90 and Hsp70 inhibition may also contribute to gene induction by removing promoter nucleosomes, as shown for galactose genes in budding yeast [354]. Currently, much more is known about the role of Hsp90 in transcriptional regulation due to a global and high-resolution chromatin-binding profile of Hsp90 by ChIP-Seq experiments [353]. Because of a strong effect of Hsp70 inhibition on transactivation potential of p65, the same approach should be performed for comparison of Hsp70 chromatin-binding with other transcriptional features.

## 4.6 Cytokine-induced conformation change of p65 occurs independently of its binding to DNA

Previous studies showed that the composition of the κB site highly regulates the activity of NF-κB dimers through conformational changes of its DNA-bound subunits. A comparison of p50/p65 dimers bound to HIV-κB, uPA (urinary plasminogen activator)-κB or IFNβ-κB sites revealed significant differences in their conformation [260]. Also, it has been suggested that the change of only one nucleotide in a κB site is enough to induce such a conformational change of a bound NF-κB that would further dictate differential assembly of co-activators to the κB site-bound dimer [259]. Interestingly, the limited proteolysis assay with TNF-stimulated $p65^{-/-}$ MEFs expressing the wild type p65 or p65 DNA-binding mutant did not detect any difference in the digestion pattern (Fig. 3.22) showing that the cytokine-induced p65 conformational change occurs independently from its contact with

DNA. It is thus possible that p65 binding to its cognate DNA causes further structural changes that serve to fine tune regulation of target gene expression.

Several recent studies showed that promoter regions of NF-κB target genes are occupied with cofactors which recruit the stimulus-induced NF-κB. ChIP experiments against CBP/p300 revealed the constitutive enrichment of this cofactor on a set of p65-dependent NF-κB target gene promoters [180]. TNF stimulation can induce recruitment of the cofactor Tip60 (60 kDA trans-acting regulatory protein of HIV type 1-interacting protein) to promoters of NF-κB target genes. Tip60 acetylates histones H3 and H4 and the resulting chromatin remodelling further leads to the recruitment of acetyl-Lys310 p65 to its target gene promoters [302]. A recent study from the Kracht lab showed that IL-1 induces CDK6 recruitment to distinct chromatin regions of inflammatory NF-κB target genes which was found to be essential for proper NF-κB p65 loading to its cognate sites [22]. Thereby it is possible that under those conditions the cytokine-induced conformational change allows proper contact of p65 with preloaded cofactors and subsequent gene expression. Phosphorylation of p65 at Ser468 leads to the SOCS-1-mediated ubiquitination and proteasome-dependent removal of promoter-bound p65 [182]. It has been shown that upon IL-1 stimulation, SOCS1 exclusively binds to DNA-bound p65 within the nucleus [211]. The interaction between the cytokine-activated p65 E39I and COMMD1 and/or Cul2 could be tested by coimmunoprecipitation. This experiment would give information whether recruitment of a COMMD1-E3 ligase complex is regulated only by a phosphorylation-mediated conformation of p65 or additional κB site-specific p65 rearrangement needs to occur. As all types of PTMs could regulate DNA-induced p65 refolding [154, 199, 214, 303], it would be interesting to learn about PTMs that exclusively occur for DNA-bound p65. It has been shown that p53 phosphorylation at Ser20 within its unstructured TAD stabilizes the interaction with p300. Dependent on the consensus DNA binding site, p300 further acetylates p53 at the C-terminal negative regulatory domain and stabilizes the p300-p53 complex [355].

Conformational changes induced by binding to consensus sites have also been observed in other transcription factors. The DBD of GR binds its cognate site in a variety of conformations and presents different interfaces for the recruitment of specific cofactors in a gene-specific manner [356-358]. Similar findings have also been reported for the POU (Pit-Oct-Unc) family of transcription factors, such as Oct-1, Oct-2, Brn-5 [359, 360]. A

recent structural study of a p53-DNA binding complex showed that a sequence specific DNA-binding occurs via an induced-fit mechanism that involves conformational switch of the p53 DBD. As many TFs bind consensus and non-consensus sequences with similar affinities, Petty *et al*. suggest that only the binding to the sequence-specific DNA induces a conformational change that locks the TF at consensus target site, controlling the TF DNA-binding independently of affinity [361]. There is no doubt that binding to DNA induces local p65 rearrangement that allow NF-κB to anchor to target κB site. It would be exciting to investigate in the future whether the allosteric effect of DNA on p65 TAD is governed by specific sequence motifs and/or dynamic chromatin organisation that changes during the process of gene transcription.

## 4.7 Non-genomic functions of NF-κB p65

After the reconstitution of p65-/- MEFs with wild type p65 and p65 DNA-binding mutants, a set of experiments was performed with the aim to characterize newly produced cells. Mutation of Glu39 to isoleucine did not affect p65 interaction with IκBα and dimerisation with p50 (data not shown). A kinetic analysis of the nuclear import and export of reconstituted MEFs revealed a rapid translocation of p65 E39I mutant into the nucleus and prolonged nuclear residence of the p65 E39I mutant (Fig. 3.21B/D). This result can be a consequence of an impaired resynthesis of p65 export factor IκBα. IκBα is a strong negative regulator of nuclear NF-κB [69]. It is fully re-synthesized within less than 60 min after TNF-stimulation, subsequently enters the nucleus and removes NF-κB from its cognate DNA [362]. However, mRNA and protein level of IκBα were not significantly affected in MEFs expressing p65 E39I, as revealed by immunoblotting with appropriate antibody and by qRT-PCR. This further suggests that mutation of E39 to isoleucine could affect the assembly of a chromosomal region maintenance-1 (Crm-1)/Exportin 1-dependent complex that mediates p65 nuclear export [44, 363]. Another possible explanation for prevented nuclear export of p65 E39I could be a disrupted interaction with the 14-3-3 protein for which is known to facilitate nuclear export of IκBα-p65 complexes [364]. TNF induces binding of p65 to 14-3-3 and their physical interaction is mediated via p65 sequences spanning residues 38-44 and 278-283 [364]. Additionally, the expression of the 14-3-3 isoform γ is controlled by p65-containing NF-κB dimers. It has been shown that binding of p65 to its consensus κB site further regulates initial induction of the 14-3-3 promoter by recruiting the TF E2A [365]. Thereby, the relative 14-3-3 mRNA amount and

protein levels should be analysed in MEFs expressing the p65 E39I mutant. Also, the interaction between p65 E39I and 14-3-3 should be tested in order to see whether the mutation of Glu39 to isoleucine disrupts the docking site for 14-3-3. Immunoblotting with phospho-specific Ser468 antibody showed prolonged phosphorylation at this site of the p65 DNA-binding mutant. As phosphorylation at Ser468 is a nuclear event [166], the prolonged phosphorylation level of p65 E39I rather reflects the impaired p65 nuclear export. Still, due to the dynamic flexibility of the overall p65 protein, the p65 E39I mutant may have a different conformation which in turn could affect the docking of kinases and phosphatases.

TNF-induced gene expression was compared between MEFs expressing wild type p65, p65 E39I or the p65 E39I/R302E mutant. Analysis of relative mRNA expression levels by qRT-PCR identified a set of p65-dependent genes that were induced only in cells reconstituted with wild type p65 (Fig. 3.23 A-E). These results show that expression of some genes is highly dependent on direct contact of p65 with its target κB site. DNA binding further leads to the proper positioning of p65 TAD and assembly of the functional transcription initiation complex. Experiments using a reconstituted cell-free systems have defined that the minimal apparatus required for an activated transcription consists of general transcription factors, RNA Pol II and a large multisubunit Mediator complex [366-368]. It was suggested that after binding to the κB site, the NF-κB dimer interacts with the Mediator complex and recruits RNA Pol II to the TSS [369, 370]. Van Essen *et al.* identified *Ip10* and *Il-6* as genes which expression depend on p65 interaction with the Mediator complex subunit Trap80, while *Mip-2* was identified as a gene independent of this interaction [369]. According to qRT-PCR experiments from this study expression of *Ip10* gene is dependent on p65 DNA-binding (Fig. 3.23D). This is supported by a previous finding that *Ip10* expression is governed by an allosteric effect of a cognate κB site [259]. Further experiments could be performed to identify to which extent the p65-Mediator complex interaction depends on p65 contact with the DNA. Also, expression of a *Mip-2* gene was governed by a p65 DNA-binding (Fig. 3.23B) and together with the finding that *Mip-2* expression is independent of p65-Trap80 interaction, this suggests that some κB site-mediated regulatory mechanism needs to occur. Putative binding sites of TFs like Sp-1, ATF/CREB, Ap-1 can be found within promoters of NF-κB target genes [369, 371-374]. Thereby, bound p65 could recruit a secondary TF that could mediate the recruitment of a

Mediator complex. As suggested by van Essen *et al.*, p65 DNA-binding may also exert its effect by inducing local chromatin alterations that would change promoter accessibility for other TF. This mechanism can be supported by study showing that in macrophages the NF-κB–driven activation of gene expression is accompanied by nucleosome remodelling at target gene promoters [375]. Also, p65 DNA-binding may induce changes to histone modifications (e.g. lysine acetylation [159, 376], lysine methylation [377] and serine phosphorylation [378, 379]) which can further control the recruitment of RNA Pol II and target gene expression.

Interestingly, another set of tested genes were expressed in cells expressing the p65 E39I mutant, suggesting that rather protein-protein interactions play a major role for their expression than a direct DNA-binding (Fig. 3.23F-J). For example, TNF-induced expression of *Il-6, Ccl9, JunB, Saa3, Cox-2* and *Nfκbiα* seems to highly depend on p65 protein-protein interactions, since the p65 E39I mutant showed a similar expression of these genes. Several studies support this finding. In a study performed by Mukherjee *et al.*, ChIP experiments identified promoters of *Nfκbiα, Tnfaip3, Cox2, Cxcl2, Csf2* and *Tnf* genes to be preloaded with the cofactor CBP/p300 [180]. In particular, RNA-Seq analysis of total mRNA isolated from TNF-stimulated MEFs reconstituted with wild type p65 or the mutant that cannot interact with CBP/p300 TAZ1 domain identified *JunB* and *Cox-2* as genes whose expression only depend on the recruitment of p65 to promoters via p65TA2-CBP/p300 TAZ1 interactions. In contrast, in the present study expression of *Tnfaip3* was observed only in cells expressing the wild type p65, suggesting that beside the interaction with CBP/p300, also the DNA-binding is necessary to occur. In a study performed by Handschick *et al.*, ChIP-Seq experiments using human KB epithelial carcinoma cells showed a tight co-recruitment of CDK6 and p65 to enhancers and promoters of many active NF-κB target genes, like *Il-8, Cxcl3, Ccl20, Il-6, Icam-1* and *Cox-2,* after the IL-1 stimulation [22]. CDK6-dependent loading of NF-κB p65 to its cognate binding sites regulates the expression of *Il-6* and *Cox-2* gene after the IL-1 stimulation. As effects on gene expression are cell- and stimulus specific, it should be tested whether the same mechanism regulates the expression of those two genes in TNF stimulated reconstituted MEFs. Another explanation for the TNF-induced gene expression in MEFs expressing the p65 E39I mutant could be the p65 interaction with the neighboring TF that is governed by a p65 phosphorylation-induced rearrangement and p50-mediated

(or by other NF-κB monomer) recruitment to DNA. It has been shown that the recruitment of RNA Pol II to *Ccl2* promoter depends on formation of a c-Jun, HDAC1/HDAC3, CBP and p65/p50 chromatin-associated protein-protein complexes at specific regions of a gene after the IL-1 stimulation. Also, this higher-order complex was not form in the absence of c-Jun [374].

Even though genes tested in this study are well documented as TNF-induced p65-dependent genes [201, 369], qRT-PCR experiments detected a slightly induced expression of *JunB* and *Nfκbiα* mRNA in p65-/- MEFs reconstituted with the empty vector (Fig. 3.23H/K). Binding sites of Sp-1, ATF/CREB and Ap-1 were identified within promoters of these two genes [369, 380]. NFAT (nuclear factor of activated T cells) and STAT TF families also bind to DNA sequences to which p65 binds [381-383]. Thus due to the absence of promoter bound p65-containing NF-κB dimers in cells reconstituted with empty vector or p65 mutants, other TF could initiate gene transcription. Different NF-κB dimers can be exchanged at the same promoter during the NF-κB response [384] and this mechanism could also be involved in the expression of genes which expression was not dependent on p65 DNA-binding. It has been shown that the *Il-6* gene is preferentially activated by a p50-IκBζ complex [385]. Still, the absence of *Il-6* expression in MEFs stably reconstituted with the double p65 E39I/R302E mutant implicates that expression of *Il-6* could be rather dependent on p65 protein-protein interactions. Analysis of the *Nfκbα* promoter structure revealed several NF-κB binding sequences. Deletion of the κB motif, located 37 bp upstream of the TATA box, specifically abolished responses to PMA and TNF [48]. The *Nfκbiα* promoter contains several potential κB sites to which p50/p65, p50/c-Rel or p65/c-Rel heterodimers as well as p50 homodimers can bind [48]. Another study revealed dynamic exchanges between members of steroid receptor cofactors (SRC) family (SRC-1, SRC-2 and SRC-3) and corepressors (SMRT, NCoR (nuclear receptor corepressor), HDAC1, HDAC2 and HDAC3) on the *Nfκbiα* promoter region during the TNF stimulation [386]. Thereby, changes in *Nfκbiα* mRNA levels detected in MEFs expressing the p65 E39I or E39I/R302E mutants could also be a consequence of impaired p65-coactivator-corepressor interactions due to the absence of a direct p65-DNA contact.

Similar experiments with DNA-binding mutants of other TFs indicated the role of protein-protein interactions for target gene expression. TF IRF3 (interferon regulatory transcription factor 3) usually exerts its function via its direct binding to IRF-binding sequences of target

genes. Reconstitution of IRF3-deficient MEFs with wild type IRF3 or its DNA-binding defective mutant (IRF3 K77E) showed that only wild type IRF3 fully restored *Tslp* and *IL33* gene induction [387]. However, IRF3 can also act as a cofactor to activate transcription of a set of NF-κB–dependent genes without directly binding to DNA [259]. The GR DNA-binding mutants (S459A and P493R) have been found to mimic a DNA-induced conformational change which allows the partial transcription of some genes. It was suggested that those DBD point mutations trigger an allosteric mechanism in which binding to the specific DNA sites normally induces the exposure of otherwise concealed GR TAD surfaces [388, 389].

ChIP-Seq experiments with reconstituted p65-/- MEFs produced in this study could be further used to reveal which regions are constitutively and/or inducible occupied by p65-containing NF-κB dimers. Such experiments would give an answer whether the DNA-binding of p65 is necessary for genomic associations and if there are regions preferentially ocupied by p65 E39I mutant. RNA-Seq experiments should be performed to confirm the effects detected by qRT-PCR since RNA-Seq experiments provides a far more precise measurement of transcript levels**.** Focusing on particular promoter regions, further ChIP and ChIP-re-ChIP experiments, using antibodies against the p65 and p50 subunits and specific transcriptional cofactors could be also performed to analyse p65 activity that is based on its protein-protein interactions. Based on qRT-PCR results, the role of a second subunit in p65-containing NF-κB dimers should be also considered and investigated. p65-/- MEFs reconstituted with p65 E39I or p65 E39I/E302R mutants would allow further investigations on the non-genomic functions of p65. Using those cells it would be possible to explore the contribution of activated, cytosolic p65-containing NF-κBs in activation of various signaling cascades, cell death and cell proliferation. The NF-κB regulatory pathway exist in mitochondria where p65-containing NF-κB dimers repress mitochondrial gene expression [258, 390]. Thereby, reconstituted MEFs used in this study would help to investigate a crosstalk between p53 and p65 in the regulation of apoptosis. The GR has been also found in the mitochondria [391]. As GR can physically interact with p65 [392], their interaction in mitochondria and regulation of processes involved in cell growth and apoptosis could be also tested. Experiments addressing these questions may reveal novel functions of p65 that do not rely on its ability to bind to DNA.

# 5. Summary

The TF NF-κB is an important regulator of immunity, stress responses as well as apoptosis, cell cycle progression and oncogenesis. NF-κB is activated by various stimuli and regulates expression many different target genes. The first part of this work shows that stimulation of cells with the cytokines TNF or IL-1 results in a profound conformational switch of the NF-κB subunit p65, as revealed by limited proteolysis assays. The cytokine-triggered reconfiguration of p65 mainly occurs for p65 contained in the nuclear fraction. Immunoprecipitation experiments with various p65-specific antibodies identified a conformation-specific monoclonal antibody that preferentially immunoprecipitates the inducibly refolded p65 protein. Cytokine inducible structural changes of p65 are mainly mediated by posttranslational p65 modifications. Mutations of individual p65 phosphorylation sites cause structural changes in p65 and thus control subsequent ubiquitination and association with transcriptional cofactors such as CBP/p300 and MYBBP1a. These experiments suggest that phosphorylation-induced conformation changes contribute to an alternative p65 configuration and thus to the specification of the NF-κB response. As chaperones are frequently found to be important for proper folding of proteins, the constitutive and inducible association of chaperones Hsc/Hsp70 and Hsp90 with p65 was tested. Coimmunoprecipitation experiments revealed constitutive interaction between p65 and Hsp70. Limited proteolysis assays showed that constitutively bound chaperones of the Hsc/Hsp70 family were not important for the cytokine-induced conformational switch *per se*, but rather control the fidelity of protein rearrangement. Accordingly, pharmacologic inhibition of Hsp/Hsc70 revealed that these chaperones also control the level of p65 modifications and regulate TNF-triggered ubiquitination and degradation of p65. A specific Hsc/Hsp70 inhibitor significantly reduced p65-triggered reporter gene activity, showing that chaperone-activity provides an important contribution for p65-induced transcription. It is thus likely that inducible conformational changes of p65 increase the intramolecular flexibility and thereby expand and specify the repertoire of possible modifications and protein-protein interactions. The cytokine-induced conformation switch is independent from the DNA-binding ability of p65, as shown by the analysis of p65-/- MEFs that were reconstituted with a p65 DNA-binding mutant. The DNA-binding mutant and a further p65 mutant additionally mutated in the NLS were used for the second part of this work. Reconstituted knockout cells were used to investigate the

contribution of p65 DNA-binding for the induction of TNF-induced and p65-dependent transcription of NF-κB target genes. Analysis of relative mRNA expression by qRT-PCR identified a set of genes which were as expected fully dependent on p65 DNA-binding. These experiments also showed that another group of genes which is independent of direct binding of p65 to its target κB sites, implicating the importance of p65 protein-protein interactions for its ability to trigger transcription. These differentially reconstituted cells will be useful in the future to identify p65-dependent non-genomic functions of NF-κB.

# 6. Zusammenfassunf

Der Transkriptionsfaktor NF-κB ist bei der Regulation der Stressantwort, der Apoptose und angeborenen Immunität beteiligt. Daher wird NF-κB durch unterschiedlichste Signale aktiviert und induziert die Expression von vielen verschiedenen Genen. Der erste Teil dieser Arbeit zeigt mit Hilfe von "limited proteolysis" Experimenten, dass die Stimulation von Zellen durch die Zytokine IL-1 oder TNF in einer Konformationsänderung des NF-κB p65 Proteins resultiert. Die Zytokin-induzierte Konformationsänderung des p65 Proteins war nur für das im Zellkern befindliche p65 Protein zu beobachten. Immunopräzipitationsexperimente erlaubten die Identifizierung eines Konformations-spezifischen p65 Antikörpers der präferentiell das umgefaltete p65 Protein erkennt. Die Zytokin-induzierten Strukturänderungen werden primär durch Phosphorylierungsereignisse am NF-κB p65 Protein induziert. Mutationen in einzelnen p65 Phosphorylierungsstellen resultierten in Strukturänderungen und kontrollierten auch weitere Eigenschaften des p65 Proteins wie beispielsweise die Ubiquitinierung und die Assoziation mit transkriptionellen Kofaktoren wie CBP/p300 und MYBBP1a. Auch die Induktion der p65 Phosphorylierung durch Behandlung der Zellen mit dem Phosphatase-Inhibitor Calyculin A führte zu einer induzierten Strukturänderung. Da Chaperone häufig an der Regulation der Proteinfaltung beteiligt sind, wurde die Interaktion von p65 mit Hsp Proteinen untersucht. "Limited proteolysis" Experimente zeigten, dass konstitutiv gebundene Hsc/Hsp70 Proteine die Genauigkeit der Zytokin-induzierten p65 Umlagerung kontrollieren. Die pharmakologische Inhibition der Hsp/Hsc70 Proteine resultierte in veränderter p65 Ubiquitinierung und Stabilität. Ebenso führte die Hsp/Hsc70 Inhibition zu einer verringerten p65-induzierten Aktivität eines Reportergens, sodass die Chaperon Aktivität einen wichtigen Beitrag für die Aktivität des p65 Proteins im Zellkern hat. Es ist wahrscheinlich, dass unterschiedliche p65 Konformationen das Repertoire an möglichen p65 Modifikationen und Protein/Protein Interaktionen stark erhöhen. Weitere Experimente zeigten, dass die Zytokin-induzierte p65 Umlagerung unabhängig von der DNA-Bindungsfähigkeit von p65 ist. Im zweiten Teil dieser Arbeit wurde die Wichtigkeit der DNA-Bindung für die Funktionen des p65 Proteins untersucht. Dazu wurden p65 knockout Zellen mit dem Wildtyp Protein sowie mit weiteren p65 Mutanten stabil rekonstituiert. Eine Mutante war DNA-Bindungsdefekt, währen die andere Mutante zusätzlich im Kerntranslokationssignal mutiert war. Die Analyse der TNF-induzierten Expression einiger NF-κB Zielgene zeigte, dass einige Gene

trotz defekter p65 DNA-Bindung noch induzierbar exprimiert werden konnten. Dieses Verhalten ist wahrscheinlich auf kompensatorische Mechanismen wie beispielsweise eine Promoter-Rekrutierung via Protein/Protein Wechselwirkungen zurückzuführen. Die differentiell rekonstituierten Zellen werden in zukünftigen Studien wichtige Informationen über DNA-Bindungs-unabhängige Funktionen des p65 Proteins liefern können.

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# Publications

- **Milanovic M**, Kracht M, Schmitz ML. The TNF-induced conformational switch of nuclear NF-κB p65 is mediated by p65 phosphorylations. Biochem J. 2014 Feb 1; 457(3):401-13.

- de la Vega, Hornung J, Kremmer E, **Milanovic M** and Schmitz ML. Homeodomain interacting protein kinase 2-dependent repression of myogenic differentiation is relieved by its caspase-mediated cleavage. Nucleic Acids Res. 2013 Jun; 41(11):5731-45.

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# Acknowledgements

I would like to express my sincere thanks to my supervisor Prof. Dr. M. Lienhard Schmitz, who gave me the opportunity to come to Germany and join his research group. His continuous guidance, expertise, enthusiasm, encouragement and support enormously helped and motivated me to keep on with my project and contributed to my development as a PhD student.

Many thanks also to Prof. Dr. Michael U. Martin for being my doctoral thesis supervisor at the Faculty of Biology and to Prof. Dr. Michael Kracht and Prof. Dr. Reinhard Dammann for joining the examination board and taking the time to go through this manuscript.

I would like to show my gratitude to my colleges and former lab members: Hilda Stekman, Julian Rodriguez, Georgette Stovall, Sharmistha Dam, Markus Sibert, Inna Grishina, Alfonso Rodriguez-Gil, Benedetto Giaimo, Francesca Ferrante, Laureano de la Vega, Tobias Wittwer, Ylia Salazar, Mercedes Lopez, Daniela Stock, Stephanie Steitz, Amit Shrestha for all the valuable suggestions and the nice discussion environment. Special thanks to Vera Saul and Ines Höfliger for tremendous help, support and positive attitude.

My sincere thanks to the 129 lab crew: Juliane Hornung, Julia Stellzig, Jan Hagenbucher, Lisa Dieterle, Carlis Farah and Rita Moreno for being there in good and bad times, dealing and shearing the happiness and craziness with me, creating the amazing working atmosphere.

I am extremely thankful to my family for the support and encouragement. Many, many thanks to Dr. Dr. Dejan Arsic, Djula Gross, Mila Milosavljevic, Jovana Cvoric, Marko Simic, Vanja Tomic, Jelena Bijelic and Svetlana Boskovic for being with me during these years.