Supplementary Materials & Methods

Antibodies, plasmids and cells

Antibodies recognizing Flag (M2, Sigma), cleaved PARP (C-2-10, Clontech), HA (3F10, Roche Molecular Biochemicals), β-actin (Abcam), p65 (C-20) and IκBα (C-21) (Santa Cruz), ubiquitin (P4D1), phosphorylated p65 and IκBα (Cell Signaling Technology) were purchased from the indicated suppliers. The COMMD1 antibody was a kind gift of Dr. Ezra Burstein (Dallas). The HA-p65 wildtype and HA-p65 S468A mutant (Mattioli *et al*, 2006), Flag-COMMD1, Flag-Cul2 and Flag-SOCS1 (Maine *et al*, 2007) were already published. (His)6-tagged ubiquitin was obtained from Dr. Daniel Krappmann (Munich). All cells were routinely cultivated in complete DMEM medium at 37°C and 5% CO_2. MEFs lacking p65 were a gift of Dr. Hiroyasu Nakano (Tokyo), IKKβ$^{-/-}$, IKKε$^{-/-}$ and IκBα$^{-/-}$ MEFs were kindly shared by Dr. Inder Verma (San Diego) Dr. Shizuo Akira (Osaka) and Amer Beg (Tampa).

Reverse transcription and real time PCR

Transfected cells were lyzed and total RNA was extracted using the RNAeasy kit (Qiagen). One µg of RNA was used for cDNA synthesis from Oligo (dT)20 primers using the Superscript first strand synthesis system (Invitrogen). Real-time PCR was performed using specific primers and SYBR Green ROX Mix (ABgene), reactions analyzed using an Applied Biosystems 7300 real time PCR system. Experiments were performed in triplicate, data were normalized to housekeeping genes and the relative abundance of transcripts was calculated by the comparative ΔΔ CT method.

Cell lysis protocols

Protein ubiquitination was measured after lyzing the cells directly in 1 x SDS sample buffer followed by sonification. Ni-NTA affinity purification was performed after lysis of cells in denaturing buffer (6 M Guanidine-HCl, 50 mM NaH_2PO_4 (pH 8.0), 10 mM Tris-HCl and 100 mM NaCl). After binding for two hours to Ni-NTA agarose (Qiagen) in the presence of 20 mM imidazole, the resin was washed under denaturing conditions and eluted as described (Roscic et al, 2006). Protein expression and protein/protein

interactions were studied in cells that were lyzed in NP-40 buffer (20 mM Tris/HCl pH 7.5, 150 mM NaCl, 1 mM phenylmethylsulfonylfluoride, 10 mM NaF, 0.5 mM sodium vanadate, leupeptine (10 μg/ml), aprotinin (10 μg/ml) and 1% (v/v) NP-40) as described (Roscic et al, 2006). For immunoprecipitation of ubiquitinated p65, 10 mM N-ethylmaleimide (N-EM) was added to the culture medium for 30 sec before washing cells in cold PBS containing 10 mM N-EM. Total cell extract was prepared with RIPA buffer supplemented with 20 mM N-EM. After centrifugation, the supernatant was further analyzed by Western blotting or immunoprecipitation.

Coimmunoprecipitation and immunoblotting

Immunoprecipitation experiments were performed by preclearance of extracts with protein A/G sepharose, followed by the addition of control or precipitating antibodies together with 25 μl of protein A/G sepharose. Samples were incubated for several hours on a spinning wheel at 4°C. The immunoprecipitates were washed 5 x with NP-40 lysis buffer and eluted by boiling in 1 x SDS sample buffer. Equal amounts of protein were separated by SDS-PAGE, followed by Western blotting to a polyvinylidene difluoride membrane (Millipore). Proteins were detected with specific antibodies by enhanced chemiluminescence as described (Hehner et al, 1999).

Pulse chase experiments

293 cells were transfected to express HA-p65 or HA-p65 S468A. The next day, cells were washed and placed in a cysteine- and methionine-deficient medium (supplemented with 10% dialyzed FCS) for 60 min. Then ^{35}S-labeled methionine and cysteine (100 μCi/ml, GE Healthcare) was added for 1 h. The labeling medium was replaced by normal DMEM supplemented with TNF and excess methionine and cysteine. At the indicated time points, cells were harvested and lysates were prepared in RIPA buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 0.5 % sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin) and the tagged p65 proteins were immunoprepipitated with αHA antibodies. After rigorous washing, bound p65 was eluted in 1 x SDS sample buffer and analyzed by SDS-PAGE and autoradiography.

ChIP assays

The following primer sequences for the detection of promoter-associated p65 were used: *Icam-1* forward: 5'-AGGGGACTAGGCAGTAGTCAATCAG-3'; reverse: 5'-GAACGAGGGCTTCGGTATTT-3' *Mip-2* forward: 5'-AGGGCAGGGCAGTAGAATGA-3'; reverse: 5'-TGTGGCTGGAGTCTGGAGTG-3'.

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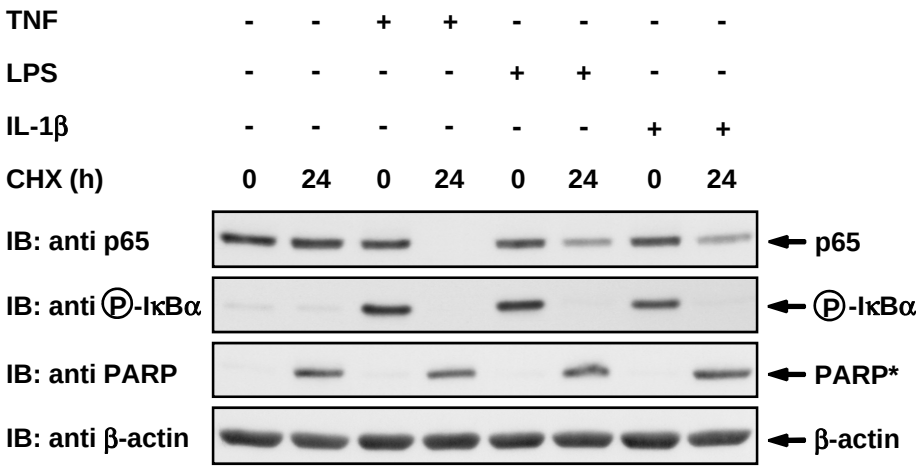
Hehner SP, Hofmann TG, Droge W, Schmitz ML (1999) The antiinflammatory sesquiterpene lactone parthenolide inhibits NF-kappa B by targeting the I kappa B kinase complex. *J Immunol* **163:** 5617-5623

Maine GN, Mao X, Komarck CM, Burstein E (2007) COMMD1 promotes the ubiquitination of NF-kappaB subunits through a cullin-containing ubiquitin ligase. *EMBO J* **26:** 436-447

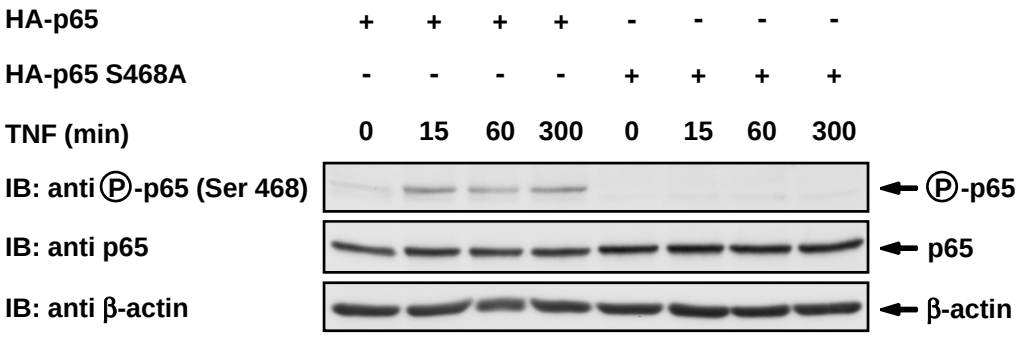
Mattioli I, Geng H, Sebald A, Hodel M, Bucher C, Kracht M, Schmitz ML (2006) Inducible phosphorylation of NF-kappa B p65 at serine 468 by T cell costimulation is mediated by IKK epsilon. *J Biol Chem* **281:** 6175-6183

Roscic A, Moller A, Calzado MA, Renner F, Wimmer VC, Gresko E, Ludi KS, Schmitz ML (2006) Phosphorylation-dependent control of Pc2 SUMO E3 ligase activity by its substrate protein HIPK2. *Mol Cell* **24:** 77-89

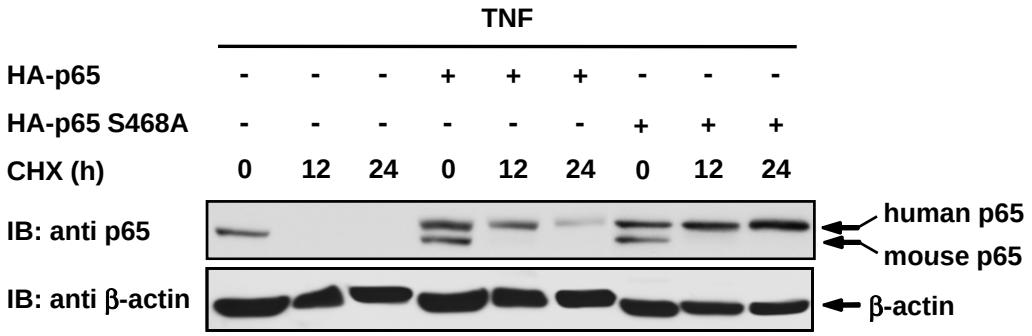
Supplementary figures



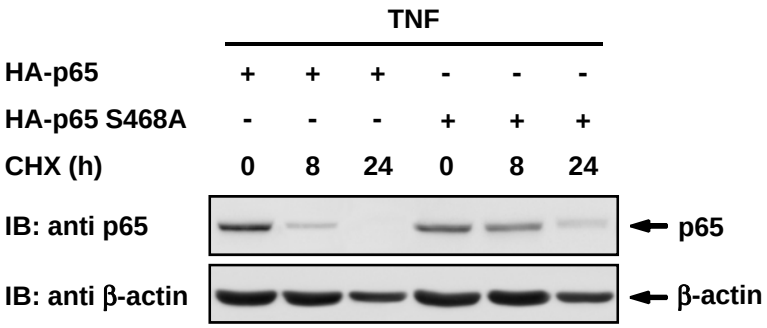
Supplementary Fig. 1. NF-κB p65 degradation in response to different stimuli. Hela cells were stimulated with TNF (15 min), LPS (30 min) or IL-1β (30 min) as shown. After washout of the stimuli, the protein synthesis blocker cycloheximide (10 μg/ml) was added for 24 hours. Equal amounts of protein contained in the total cell extracts were analyzed by immunoblotting (IB) for the occurrence of p65, the caspase-cleaved PARP fragment (indicated by PARP*) and β-actin as shown. Phosphorylation of IκBα was revealed by a phospho-specific antibody.



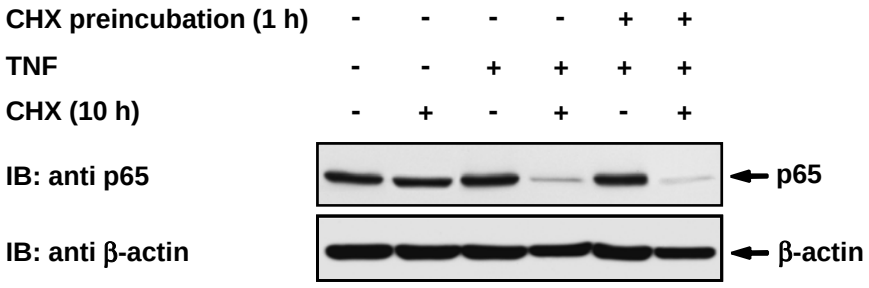
Supplementary Fig. 2. Phosphorylation of p65 at serine 468 by TNF. Cells were transfected as shown and subsequently stimulated for the indicated periods with TNF, followed by cell lysis and analysis of p65 phosphorylation by immunoblotting.



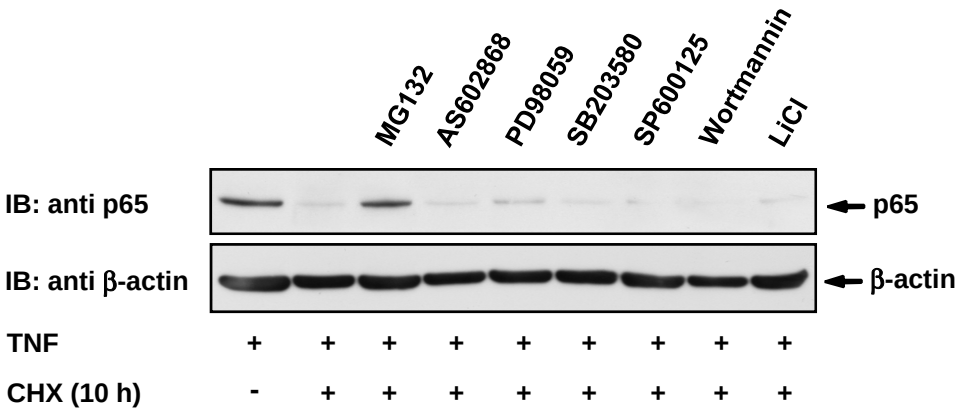
Supplementary Fig. 3. p65 stability in IκBα$^{-/-}$ cells. MEFs lacking IκBα were transfected with empty vector or the indicated p65 expression vectors as shown, followed by the analysis of p65 stability with the protocol described in (S1). The positions of human and mouse p65 are indicated.



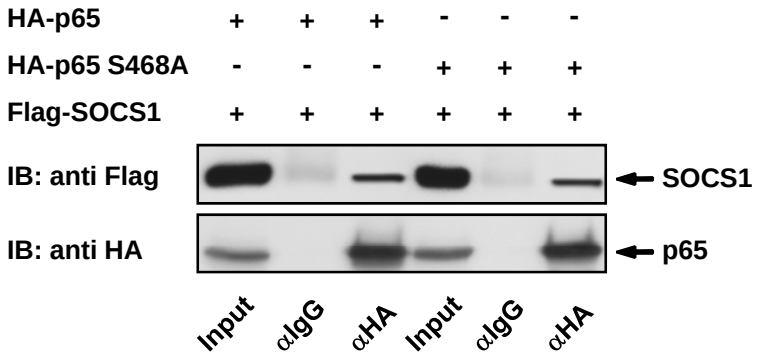
Supplementary Fig. 4. Kinetics of p65 decay. $p65^{-/-}$ MEFs were transiently transfected to express HA-p65 or the HA-p65 S468A mutant as shown. 36 hours later, cells were stimulated with a 15 min pulse of TNF, followed by the addition of cycloheximide for the indicated periods. Total cell extracts were tested by immunoblotting for p65 and β-actin protein levels.



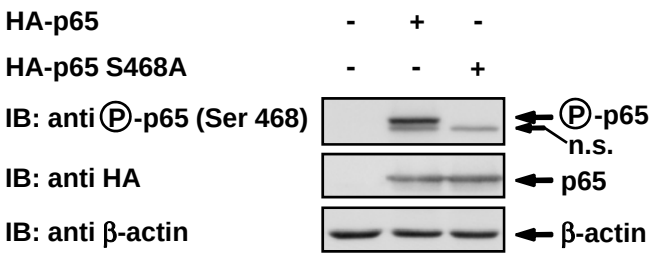
Supplementary Fig. 5. p65 degradation is independent from protein synthesis. IκBα$^{-/-}$ MEFs were pulsed for 15 min with TNF and further incubated with CHX as shown. In one experiment, cells were preincubated for 1 h with CHX prior to TNF treatment in order to inhibit protein synthesis during TNF stimulation. Protein levels were revealed by immunoblotting.



Supplementary Fig. 6. Analysis of signaling pathways leading to p65 degradation. TNF-induced elimination of the endogenous p65 protein was triggered in IκBα$^{-/-}$ MEFs as described in (S1), but cells were pretreated for 1 h with the following inhibitors: MG132 (proteasome inhibitor, 10 μM), AS602868 (IKK inhibitor, 12 μg/ml), PD98059 (ERK inhibitor, 50 μM), SB203580 (p38 MAPK inhibitor, 20 μM), SP600125 (JNK inhibitor, 20 μM), Wortmannin (PI3K inhibitor, 2 μM) or LiCl (GSK3 inhibitor, 25 mM) as shown. Cell extracts were further analyzed by immunoblotting using the indicated antibodies.



Supplementary Fig. 7. Interaction of p65 with SOCS1. 293T cells were transiently transfected with HA-tagged p65 proteins either alone or together with Flag-tagged SOCS1 as shown. Following immunoprecipitation with either HA or control IgG antibodies, precipitated proteins were revealed by immunoblotting.

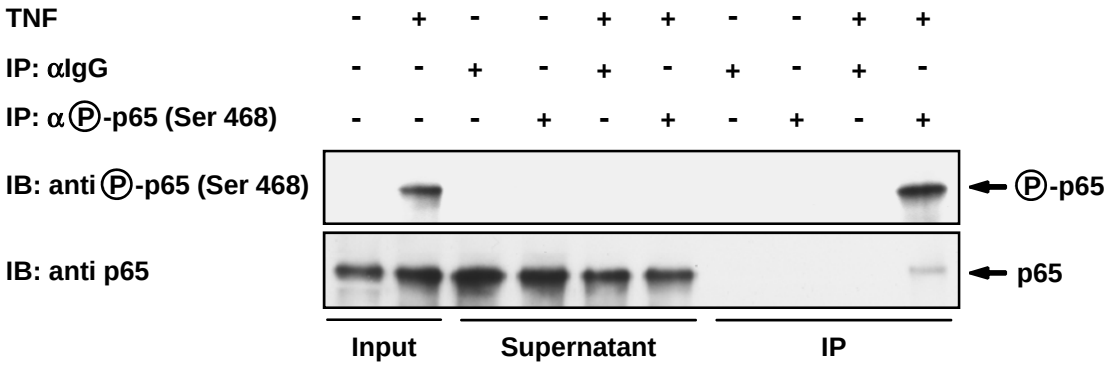


Supplementary Fig. 8. Analysis of p65 serine 468 phosphorylation. Cells were transfected as shown and lyzed in NP-40 buffer. Extracts were further analyzed for the phosphorylation of p65 by immunoblotting. n.s. = non-specific band.

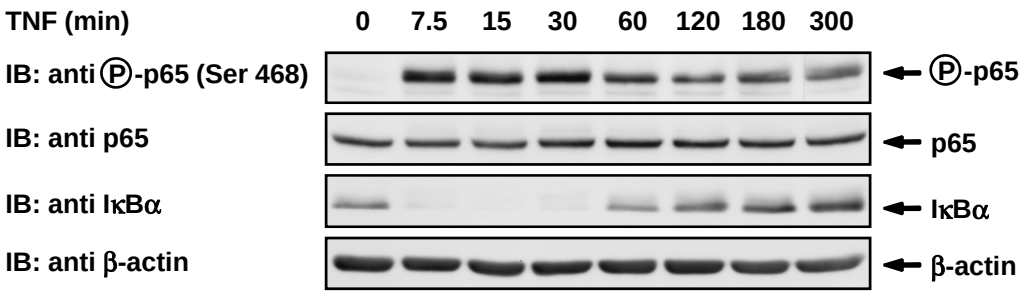
Microarray results for p65-dependent genes

Group	Induction by p65 reconstitution	Kinetics during stimulation	Gene symbol	Synonym	ReSeq Accession
A	p65 wt ≈ p65 S468A	wt ≈ S468A 8 h ≈ 1.5 h	Cxcl2 C3 Ccl5 Gch Vcam1	MIP-2 RANTES 	NM_009140 NM_009778 NM_013653 NM_008102 NM_011693
B		wt ≈ S468A 8 h < 1.5 h	Ccl2 Cxcl1 Cxcl10 Nfkbia	MCP-1 GRO1 IP-10 	NM_011333 NM_008176 NM_021274 NM_010907
C	p65 wt > p65 S468A	wt ≈ S468A 8 h > 1.5 h	Mmp13 Mmp3		NM_008607 NM_010809
D		wt ≈ S468A 8 h ≈ 1.5 h	Gpr84 Saa3		NM_030720 NM_011315
E	p65 wt < p65 S468A	wt ≈ S468A 8 h ≈ 1.5 h	Csf2 Sele	GM-CSF ELAM-1	NM_009969 NM_011345
F		wt: 8 h < 1.5 h S468A: 8 h ≈ 1.5 h	Icam1		NM_010493

Supplementary Fig. 9. Kinetic analysis of gene expression induced by p65 or p65 S468A. p65-deficient cells were retransfected with either empty vector, p65 wildtype (wt) or the p65 S468A mutant. The next day, cells were left untreated or stimulated for 1,5 or 8 h with TNF, followed by RNA extraction and microarray analysis on a mini-array representing selected inflammatory genes. Only the p65-dependent genes are shown in this table. The regulated genes are grouped according to the relative induction by wildtype versus mutant and by their kinetic behaviour. A threshold value of 1.5-fold relative gene expression change was set and provided the basis for grouping of genes and kinetics as depicted in the table.



Supplementary Fig. 10. Quantitative analysis of p65 phosphorylation. 293 cells were left untreated or stimulated for 60 minutes with TNF. Cell extracts were used for immunoprecipitation (IP) with antibodies recognizing serine 468 phosphorylated p65. A sample of the input material and IP supernatant were kept for further Western blot analysis. The IP was further washed and the eluates were analyzed by immunoblotting together with the material representing the input and supernatant for the occurrence and phosphorylation of p65. The results show efficient IP of the phosphorylated p65 protein but large amounts of unmodified p65 in the supernatant.



Supplementary Fig. 11. Kinetic analysis of p65 serine 468 phosphorylation. MEFs were treated with TNF for the indicated periods, followed by the analysis of p65 phosphorylation and IκBα degradation by immunoblotting with specific antibodies.