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SIRT2 regulates NF-κB-dependent gene expression through deacetylation of p65 Lys310

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Summary

NF-κB regulates the expression of a large number of target genes involved in the immune and inflammatory response, apoptosis, cell proliferation, differentiation and survival. In this study, we identified SIRT2 as a deacetylase of the transcription factor p65. SIRT2 is a member of the family of sirtuins, which are NAD^+-dependent deacetylases involved in several cellular processes. SIRT2 interacts with p65 in the cytoplasm and deacetylates p65 in vitro and in vivo at Lys310. Moreover, p65 is hyperacetylated at Lys310 in *Sirt2*$^{-/-}$ cells after TNFα stimulation, which results in the increase in expression of a subset of p65 acetylation-dependent target genes. Our work provides evidence that p65 is deacetylated by SIRT2 in the cytoplasm to regulate the expression of specific NF-κB-dependent genes.

Key words: NF-κB, SIRT2, Acetylation, p65, RelA, K310

Introduction

Nuclear factor kappa B (NF-κB) belongs to a family of inducible transcription factors that modulates gene expression in response to a variety of extracellular and intracellular stimuli (Bonizzi et al., 2004; Hoffmann and Baltimore, 2006; Perkins, 2006). NF--κB has a crucial role in the regulation of many genes involved in mammalian immune and inflammatory response, such as cytokines, cell adhesion molecules, complement factors and a variety of immune receptors. It has additionally been implicated as an important regulator of cellular processes such as apoptosis, cell proliferation and differentiation. The mammalian NF-κB family includes five members, p65 (RelA), c-Rel, RelB, p105/p50 (NF-κB1) and p100/p52 (NF-κB2), encoded by *RELA*, *REL*, *RELB*, *NFKB1* and *NFKB2*, respectively (Hayden and Ghosh, 2008). These proteins form homo- or heterodimers. The most abundant heterodimer in the majority of cells consists of the two subunits p65 and p50.

In most unstimulated cells, NF-κB is sequestered in the cytoplasm as an inactive transcription factor complex through physical association with one of the inhibitors of NF-κB (IκB, predominantly IκBα). NF-κB induction involves the rapid activation of IKKβ- and NEMO-dependent phosphorylation and subsequent degradation of IκB proteins. Consequently, dissociation of IκB proteins from NF-κB unmasks the nuclear localization sequence (NLS) of p65, which leads to nuclear translocation and binding of NF-κB to specific κB consensus sequences in the chromatin, to regulate specific subsets of genes (Ghosh et al., 1998; Pahl, 1999). Interestingly, one consequence of NF-κB activation is the upregulation of IκBα gene expression, mediated by a κB consensus sequence within the IκBα promoter (Perez et al., 1995). Several lines of evidence suggest that newly synthesized IκBα enters the nucleus, displaces non-chromatin associated subunits of NF-κB, thereby mediating their export from the nucleus and re-establishing a cytoplasmic pool of inhibited complexes (Huang et al., 2000). Relocation of NF-κB into the cytoplasm and attenuation of NF-κB-mediated transcriptional activation therefore provides a feedback mechanism for modulating the extent and duration of inflammatory responses by cells. Nevertheless, if cells are continuously exposed to a specific stimulus, NF-κB translocates back to the nucleus some time after the first wave of NF-κB induction to continue with transcriptional regulation of target genes (Hoffmann et al., 2002). The duration of the NF-κB response depends at least in part on the stimulus applied and on the type of cell.

NF-κB specificity is regulated at different levels in the cell (Perkins et al., 1997). It is plausible that different stimuli induce distinct post-translational modifications of p65, determining the outcome of p65-mediated transcriptional events. Several post-translational modifications on p65 have been described, the most important ones being phosphorylation and acetylation (Perkins, 2006). Phosphorylation of p65 enhances its interaction with its transcriptional coactivator CBP/p300 and with components of the general transcription machinery (Zhong et al., 2002). Acetylation of p65 was reported to modulate its DNA-binding capacity, transcriptional activity, interaction with IκB proteins and subcellular localization (Chen et al., 2001; Kiernan et al., 2003). We recently showed that p65 is acetylated by p300 not only at Lys310 (K310), but also at K314 and K315, two novel acetylation sites, and that acetylation of K314 is important for late gene expression (Buerki et al., 2008; Rothgiesser et al., 2010). Microarray analysis of genetically complemented p65 knockout (–/–) cells treated with tumor necrosis factor α (TNFα) identified specific sets of genes differentially regulated by acetylation-deficient mutants of p65 compared with wild-type cells. Together, these results show that site-specific p300-mediated acetylation of p65 regulates the specificity of NF-κB-dependent gene expression.

Reversible protein acetylation is an important post-translational modification that regulates the function of histones and many other

proteins (Sterner and Berger, 2000). Acetylation has a rapid turnover because of the highly dynamic equilibrium between two different groups of enzymes: histone acetyltransferases (HATs) and histone deacetylases (HDACs). The balance between these two activities is key to regulate the appropriate cellular response to signals. Mammalian HDACs are divided into four main classes based on sequence similarity and cofactor dependency (class I–IV) (Yang and Seto, 2007). Class III HDACs, also known as sirtuins, constitute a special class of enzymes because of their requirement for NAD^+ as a cofactor in their deacetylation reaction. During the deacetylation reaction, nicotinamide (Nam) is cleaved from NAD^+ and the acetyl group of the substrate is then transferred to ADP ribose, generating the novel metabolite 2′-O-acetyl-ADP ribose (OAADPr) (Denu, 2003). The sirtuin family consists of seven members in mammals (SIRT1–SIRT7) that contain a conserved catalytic core domain comprised of approximately 275 amino acids. They regulate a variety of cellular functions, such as genome maintenance, longevity, and metabolism (Oberdoerffer and Sinclair, 2007; Westphal et al., 2007; Yamamoto et al., 2007). Until now, all sirtuins, except for SIRT4, have been described to have deacetylation (Taylor et al., 2008). The SIRT proteins exhibit differential localization from nucleus to nucleolus, and from cytoplasm to mitochondria (Sauve et al., 2006).

SIRT2 is the counterpart of yeast Hst2p and both proteins localize to the cytoplasm (Perrod et al., 2001). SIRT2 was found to bind HDAC6 and to deacetylate α-tubulin, thereby participating in the regulation of microtubule dynamics and possibly cell cycle progression (Dryden et al., 2003; North et al., 2003; Pandithage et al., 2008). Presumably, SIRT2 regulates other cellular functions, because p53, p300 and histones (H3 and H4) have been identified as substrates (Black et al., 2008; Heltweg et al., 2006; Vaquero et al., 2006). The apparent contradiction of a protein being cytoplasmically located, yet exhibiting specificity for a histone residue was resolved when SIRT2 localization was monitored during cell cycle progression. SIRT2 localizes to the cytoplasm throughout the cell cycle, with the exception of prophase during G2–M transition, where it translocates to the nucleus to deacetylate histone H4 at Lys16 (H4K16) (Vaquero et al., 2006). Moreover, MEFs derived from *Sirt2*$^{-/-}$ mice show hyperacetylation of H4K16 during mitosis (Vaquero et al., 2006). In addition, the dynamic analysis of the subcellular localization of SIRT2 revealed that this enzyme shuttles continuously between the cytoplasmic and nuclear compartments during interphase (North and Verdin, 2007).

Based on our recent findings, we extended our studies to further elucidate the molecular regulation of p65 acetylation. Here, we describe the characterization of SIRT2 as a deacetylase of p65. Furthermore, gene expression analysis in SIRT2-deficient MEFs indicates that SIRT2-mediated p65 deacetylation is implicated in transcriptional regulation of a subset of genes.

Results

Generation and verification of a specific antibody against acetylated p65 K310

To further assess the functional relevance of p65 acetylation in vivo, we generated antibodies raised against acetylated K310. The selected antibody recognized specifically p65 acetylated in vitro and in cells by p300 at K310 (Fig. 1A,B). Furthermore, we investigated the kinetics of p65 acetylation in response to TNFα stimulation in cells. *p65*$^{-/-}$ MEFs stably complemented with p65 (Buerki et al., 2008) were stimulated for the indicated times (20–90 minutes). Western blot analysis of immunoprecipitated p65 from the nuclear fraction of the cells revealed that endogenous p65 predominantly translocated after 20 minutes of TNFα treatment into nucleus, which correlated with its acetylation at K310 (Fig. 1C). The signal dramatically decreased after 45 minutes of stimulation and was no longer detectable after 90 minutes, which correlates with the reported cytoplasmic relocalization of p65 in MEFs after TNFα stimulation (Hoffmann et al., 2002). We also performed the reciprocal experiment in *p65*$^{-/-}$ MEFs stably complemented with wild-type (wt) and K310R p65. Immunoprecipitation with anti-acetylated K310 (anti-acK310) antibody from the nuclear extracts of TNFα-stimulated cells and subsequent western blot analysis with an anti-p65 antibody showed again that p65 was rapidly acetylated 20 minutes after TNFα stimulation (Fig. 1D). Acetylation took place at K310 and was specific, because the corresponding K310 acetylation mutant cells displayed no acetylation signal at K310 on p65. Together, these experiments revealed that p65 is acetylated at K310 in a TNFα-dependent manner, possibly upon translocation to the nucleus.

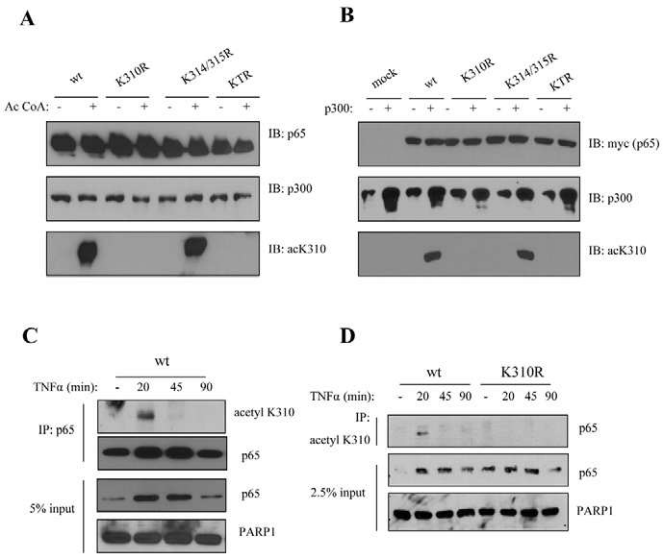

Fig. 1. Kinetics of p65 K310 acetylation upon TNFα stimulation. (**A**,**B**) Characterization of specific antibody against p65 acetylated at K310. (A) Purified recombinant p65 wild type and the acetylation-deficient mutants were incubated with recombinant p300 in the presence (+) or absence (–) of acetyl CoA. Proteins were resolved by SDS-PAGE and analyzed by western blot using the indicated antibodies. (B) HEK293T cells were transfected with p65 wild type or mutants, with (+) or without (–) p300 co-transfection. Acetylation of p65 at specific K310 was assessed by western blot using the specific antibody. (**C**,**D**) Endogenous p65 is rapidly and possibly transiently acetylated in the nucleus upon TNFα stimulation in vivo. (C) Wild-type p65 complemented MEFs were treated with TNFα for the indicated time periods. p65 was immunoprecipitated from nuclear extracts and analyzed by western blot using anti-acetyl K310 antibody. PARP1 from 5% input was used as nuclear loading control. (D) Wild-type or K310R p65 complemented MEFs were treated with TNFα for the indicated time periods. Acetyl K310 was immunoprecipitated from nuclear extracts and analyzed by western blot using anti-p65 antibody. PARP1 from 2.5% input was used as nuclear loading control.

p65 is deacetylated by SIRT1 and SIRT2 at K310 in vitro

The reversed acetylation of p65 prompted us to investigate which enzyme might be responsible for p65 deacetylation at K310. Here we focused on sirtuin proteins. To elucidate which sirtuin can

deacetylate p65 at K310, we purified (baculovirus expressed) recombinant SIRT1, SIRT2, SIRT6 and SIRT7, which are known to localize either to the nucleus or to the cytoplasm (Fig. 2A). Recombinant purified p65 was acetylated in vitro by p300 and subsequently incubated with purified recombinant SIRT1, SIRT2, SIRT6 and SIRT7, in the presence or absence of NAD^+. Although SIRT2 was able to deacetylate p65 completely after 30 minutes, as observed by western blot analysis using the anti-acK310 antibody, SIRT1 was only able to do so after extended incubation (Fig. 2B and supplementary material S1A). SIRT6 and SIRT7 were not able to deacetylate p65 under the tested conditions.

p65 is deacetylated by SIRT2 at K310 in vivo

Since SIRT1 has been previously reported to deacetylate p65 (Yeung et al., 2004), we decided to focus on SIRT2. To confirm that SIRT2 is also able to deacetylate p65 in vivo, His-tagged SIRT2 and SIRT6 or SIRT7 (as negative controls) were overexpressed in HEK293T cells (supplementary material Fig. S1B), along with Myc-tagged p65. Stimulation of transfected cells with TNFα for 30 minutes resulted in effective acetylation of p65 K310 (Fig. 2C, mock sample). Notably, overexpression of SIRT2 significantly reduced p65 acetylation at K310, whereas SIRT6 and SIRT7 were not able to deacetylate p65 under these conditions (Fig. 2C). Similarly, overexpressing class I HDACs HDAC1, HDAC2 and HDAC3 was insufficient to deacetylate K310 of p65 (supplementary material Fig. S1C,D). To verify that the deacetylase activity of SIRT2 was necessary for p65 deacetylation, we overexpressed the wt and a catalytically inactive mutant of SIRT2 (H187Y), with p65 in HEK293T cells (Fig. 2D). The enzymatically inactive SIRT2 mutant was, as expected, unable to deacetylate p65 at K310. These findings indicate that SIRT2 is able to deacetylate K310 of p65 in cells. We have previously shown that p65 is acetylated by p300 at K314 and K315 (Buerki et al., 2008). We also tested whether SIRT2 was able to deacetylate p65 at these residues. SIRT2 could deacetylate p65 at K314 and K315 in vitro and in HEK293T cells when overexpressed with p65 and p300 (supplementary material Fig. S1E,F). However, antibodies raised against acetylated K314 and K315 did not recognize endogenous p65 and therefore we concentrated on K310 acetylation of p65 for further investigations.

TNFα stimulation does not induce nuclear translocation of SIRT2

Next, we decided to investigate whether SIRT2 translocates to the nucleus upon TNFα stimulation. Since endogenous SIRT2 could not be detected by immunofluorescence, we overexpressed HA-tagged SIRT2 in HeLa cells and stimulated the cells with TNFα for 30 or 90 minutes. Immunofluorescence studies revealed that overexpressed SIRT2 and endogenous p65 localized to the cytoplasm in untreated cells (Fig. 3A). After 30 minutes of TNFα stimulation, p65 translocated to the nucleus and relocated to the cytoplasm after 90 minutes, whereas SIRT2 remained in the cytoplasm throughout the experiment, indicating that SIRT2 does not shuttle to the nucleus upon TNFα stimulation (Fig. 3A).

SIRT2 forms a complex with p65 in the cytoplasm, which is enhanced by TNFα stimulation

To investigate whether endogenous SIRT2 and p65 interact in vivo, p65 was immunoprecipitated from cytoplasmic or nuclear extracts of THP1 cells. Interestingly, SIRT2 coimmunoprecipitated with p65 in the cytoplasmic extracts under basal conditions, indicating that p65 forms a complex with SIRT2 in untreated cells

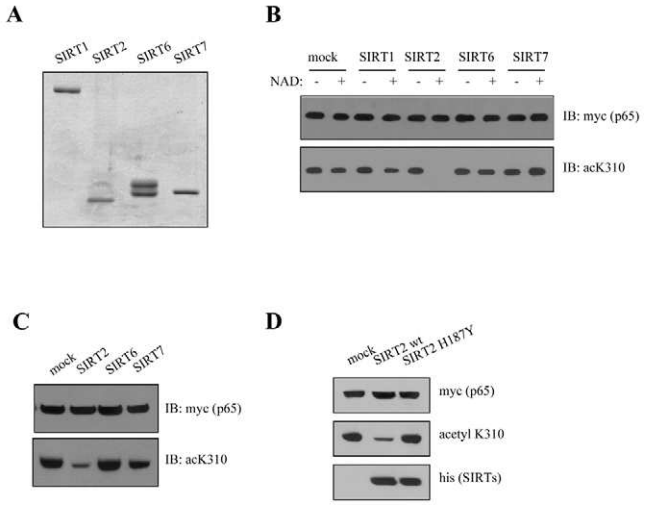

Fig. 2. SIRT2 deacetylates p65 at K310. (**A**) Expression and purification of His-tagged SIRT1, SIRT2, SIRT6 or SIRT7 from insect cells. (**B**) In vitro acetylated p65 wild type was incubated for 30 minutes with recombinant sirtuins in the presence (+) or absence (–) of NAD^+. Deacetylation was confirmed by western blot analysis using the acK310-specific antibody. (**C**) Overexpressed SIRT2 deacetylates p65 at K310 in HEK293T cells. HEK293T cells were transfected with the expression plasmids for the indicated His-tagged SIRT proteins with p300 and Myc-tagged p65. Whole-cell extracts were prepared after 30 minutes of TNFα stimulation and analyzed by western blot. (**D**) Deacetylase activity of SIRT2 is required for p65 deacetylation in cells. SIRT2 wild type or the enzymatic inactive mutant was overexpressed in HEK293T cells with p300 and Myc-tagged p65. The acetylation status of p65 after 30 minutes of TNFα stimulation was assessed with the indicated antibody.

(Fig. 3B). Although TNFα stimulation induced nuclear translocation of p65, enhanced p65–SIRT2 complex formation was observed in the cytoplasmic fraction (Fig. 3B). SIRT2 did not immunoprecipitate with nuclear p65, confirming that TNFα did not induce SIRT2 translocation.

SIRT2 deacetylates p65 at K310 in the cytoplasm

To provide further evidence that SIRT2 deacetylates p65 in the cytoplasm, HEK293T cells were transfected with expression plasmids for different SIRT2 mutants containing an additional NLS or an NLS in combination with a NES deletion (NLSΔNES). Whereas wild-type SIRT2 was located exclusively in the cytoplasm, fusion of an NLS to SIRT2 slightly increased its nuclear localization (Fig. 3C). Complete nuclear localization of SIRT2 could be observed only upon additional deletion of the NES (NLSΔNES mutant). Importantly, analysis of the p65 acetylation status under these conditions revealed that the nuclear localization of SIRT2 negatively correlated with p65 deacetylation (Fig. 3D). The mutation of SIRT2 to achieve nuclear localization (SIRT2 NLSΔNES mutant) did not abolish its deacetylation activity, because it was able to efficiently deacetylate H4K16 in the nucleus (supplementary material Fig. S2A,B). Collectively, these results suggest that SIRT2 interacts with and deacetylates p65 exclusively in the cytoplasm.

Hyperacetylation of p65 at K310 in *Sirt2*$^{-/-}$ cells

To further investigate the role of SIRT2 in p65 deacetylation in vivo, *Sirt2*$^{-/-}$ and *Sirt2*$^{+/+}$ MEFs were stimulated with TNFα and

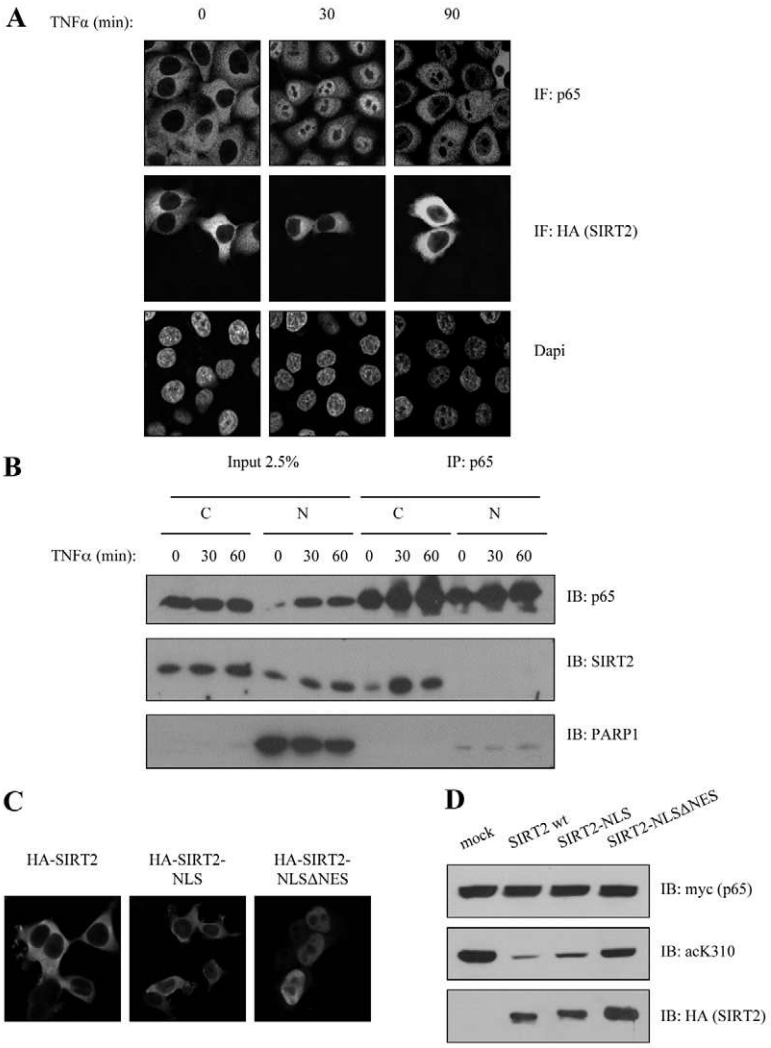

Fig. 3. SIRT2 deacetylates p65 in the cytoplasm. (**A**) p65 translocates to the nucleus in response to TNFα stimulation, whereas SIRT2 stays in the cytoplasm. HeLa cells were transfected with HA-tagged SIRT2 and stimulated with TNFα for the indicated time points, followed by immunofluorescence detection of p65 and HA-SIRT2. Nuclei are counterstained with DAPI. Subcellular localization of p65 and HA–SIRT2 was analyzed with a confocal microscope. (**B**) p65 interacts with SIRT2 in the cytoplasm. THP1 cells were stimulated with TNFα for the indicated time points. p65 was immunoprecipitated from nuclear and cytoplasmic extracts and analyzed by western blot using indicated antibodies. Levels of PARP1 as nuclear control are shown. (**C**) HEK293T cells were transfected with HA-tagged SIRT2 wild type, NLS mutant or NLSΔNES mutant. After 30 minutes of TNFα stimulation, cells were fixed and immunostained with anti-HA antibody to display cellular localization of SIRT2 wild type and mutant proteins, assessed with a confocal microscope. (**D**) Strongly reduced decatylation of p65 acK310 upon overexpression of nuclear SIRT2 mutant. The same constructs as in C were overexpressed in HEK293T cells along with p65 wild type and p300, followed by 30 minutes of TNFα stimulation, whole-cell extracts preparation and western blot analysis with the indicated antibodies.

acetylation of endogenous p65 was analyzed by western blot. The increase in acetylation of endogenous p65 at K310 upon TNFα stimulation was substantially larger in *Sirt2*$^{-/-}$ compared with wild-type MEFs (Fig. 4A). Protein levels of SIRT1 were not altered in the analyzed MEFs (Fig. 4B). Moreover, *Sirt1*$^{-/-}$ MEFs did not display hyperacetylated p65 (supplementary material Fig. S3A). Together, these results provide strong evidence that SIRT2 is the predominant enzyme involved in deacetylation of p65 at K310 after TNFα stimulation in vivo.

SIRT2 regulates NF-κB-dependent gene expression

Since p65 acetylation influences NF-κB-dependent transcription, we decided to investigate whether depletion of SIRT2 affects the expression of NF-κB target genes upon TNFα stimulation in vivo. We analyzed the expression of *Mpa2l*, a gene previously identified to be dependent on NF-κB (Rothgiesser et al., 2010), which requires acetylation of p65 at K310 for its transcriptional activation (supplementary material Fig. S3B). Gene expression analysis by real-time RT-PCR in *Sirt2*$^{+/+}$ and *Sirt2*$^{-/-}$ MEFs uncovered a dramatic increase in *Mpa2l* expression in *Sirt2*$^{-/-}$ cells compared with *Sirt2*$^{+/+}$ cells in response to TNFα (70-fold difference, Fig. 4C). The same was not observed for the control *Rela,* a gene that has not been reported to be dependent on NF-κB or to be a housekeeping gene (www.nf-kb.org) (Fig. 4D). Moreover, knockdown of *p65* in *Sirt2*$^{-/-}$ cells reduced the induction of *Mpa2l* expression (supplementary material Fig. S3C,D). Transient knockdown of *Sirt2* with siRNA in MEFs also enhanced *Mpa2l* expression, although not to the same extent as in *Sirt2*$^{-/-}$ cells (Fig. 4E,F). This correlated with our results showing that acetylation of p65 at K310 is required for transcriptional activation of *Mpa2l* (supplementary material Fig. S3B) and that p65 is hyperacetylated in *Sirt2*$^{-/-}$ MEFs (Fig. 4A). In addition, we tested whether more NF-κB target genes, other than *Mpa2l*, were expressed to a greater extent in *Sirt2*$^{-/-}$ cells upon TNFα stimulation. Real-time RT-PCR analysis of 12 more NF-κB target genes, revealed that half of the tested genes were expressed at least twofold more in *Sirt2*$^{-/-}$ cells than in *Sirt2*$^{+/+}$ cells upon TNFα stimulation (Table 1). These data imply that there is a subset of NF-κB target genes that are particularly dependent on K310 acetylation and that inability to deacetylate p65 K310 is associated with greatly increased gene induction, as observed in *Sirt2*$^{-/-}$ cells.

Sirt2$^{-/-}$ MEFs display resistance to TNFα-induced apoptosis

Finally, we questioned the physiological relevance of enhanced gene expression in *Sirt2*$^{-/-}$ MEFs after TNFα stimulation. p65$^{-/-}$ MEFs are sensitive to TNFα-induced apoptosis (Beg and Baltimore,

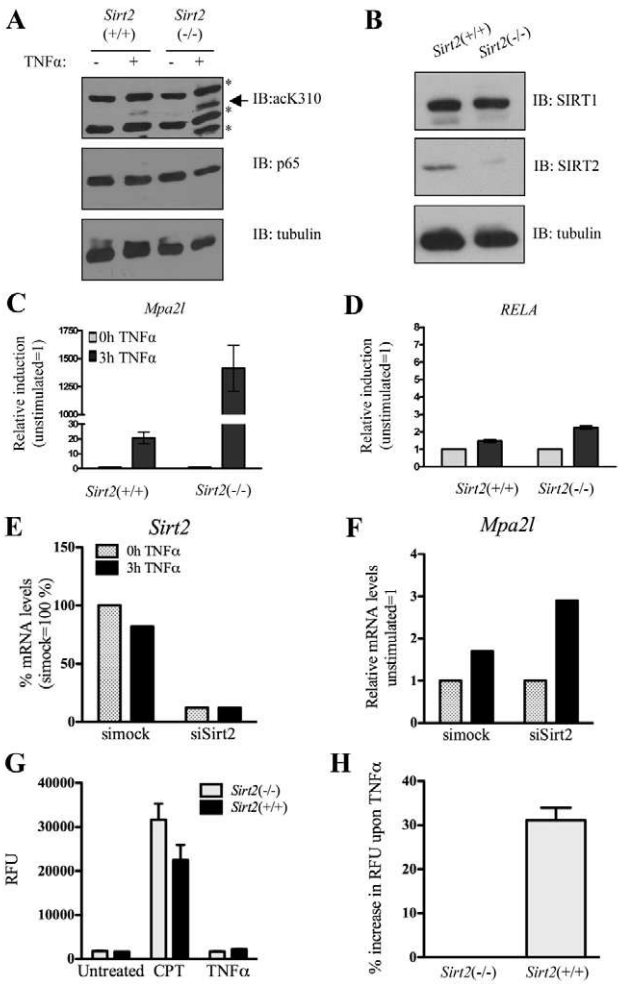

Fig. 4. Lack of SIRT2 increases TNFα-induced NF-κB-dependent transcriptional activation. (**A**) Endogenous p65 is hyperacetylated in *Sirt2*$^{-/-}$ cells. MEFs from the indicated genotypes were pre-treated with the HDAC inhibitors (HDACi) TSA and Nam for 30 minutes and then stimulated with TNFα for 1 hour before preparing whole-cell extracts. Extracts were analyzed by western blot using anti-acK310 antibody. The membrane was reprobed for p65, and tubulin was used as loading control. * indicates a non-specific band. (**B**) Western blot analyses for endogenous SIRT1 and SIRT2 in *Sirt2*$^{-/-}$ cells. Tubulin was used as loading control. Gene induction of *Mpa2l* (**C**) and *Rela* (**D**) in *Sirt2*$^{+/+}$ and *Sirt2*$^{-/-}$ MEFs after 3 hours of TNFα stimulation, as measured by real-time RT-PCR. At least two biological replicates were analyzed. Shown are the means ± s.d. of three independent runs. (**E**,**F**) Knockdown of *Sirt2* increases gene induction of *Mpa2l*. *p65*$^{-/-}$ MEFs stably complemented with wild-type p65 were transiently transfected with the indicated siRNA oligos, stimulated with TNFα for 3 hours and *Sirt2* levels (E) and gene induction of *Mpa2l* (F) was analyzed by real-time RT-PCR. (**G**,**H**) Caspase-3 activity assay in *Sirt2*$^{-/-}$ and *Sirt2*$^{+/+}$ MEFs. Cells were treated with one of the following: 100 ng/ml TNFα or 50 μM Camptothecin. After 20 hours the cells were harvested by slow speed centrifugation and analyzed for the Caspase-3 activity assay using a plate reader. The experiment was performed twice and results from one experiment performed in triplicate are shown. (G) RFU relative signals as obtained from the plate reader. (H) Percentage increase in RFU calculated as increase in signal after TNFα treatment relative to the untreated sample.

1996). This encouraged us to stimulate *Sirt2*$^{-/-}$ and *Sirt2*$^{+/+}$ MEFs with TNFα (100 ng/ml) and measure Caspase-3 activity as a marker for apoptosis. We used Camptothecin (CPT) (50 μM) as a positive control. Stimulation of cells with CPT lead to significant increase in Caspase-3 activity and was at least 15 times more than the signal obtained from TNFα-stimulated samples (Fig. 4G). There was no significant difference between the cell lines. However, stimulation with TNFα led to a differential response. Whereas *Sirt2*$^{+/+}$ cells displayed a ~30% increase in Caspase-3 activity upon TNFα induction, *Sirt2*$^{-/-}$ cells were protected from TNFα-induced Caspase-3 activation (Fig. 4H). These results suggest that *Sirt2*$^{-/-}$ cells are protected from TNFα-induced apoptosis and this might be due to enhanced NF-κB gene induction, which we observed in these cells (Table 1).

Table 1. Fold induction of NF-κB target genes upon TNFα stimulation[a]

	Sirt2$^{-/-}$	*Sirt2*$^{+/+}$
Cxcl5	318	46
Ccl20	488	56
Il6	26	4
Ip10	21083	75
Mmp9	63	9
Mmp13	4.7	1.2
Cxcl2	36	31
Cox2	1.3	0.9
Ikba	20.3	15
Mcp1	913	693
Lif	20.7	11.4
Gadd45b	1.9	2.9

[a]*Sirt2*$^{-/-}$ and *Sirt2*$^{+/+}$ MEFs were stimulated with TNFα for 3 hours and gene expression was measured by real-time RT-PCR. Results are means of three independent experiments.

Discussion

We have previously shown that p65 acetylation has an important role in the regulation of NF-κB-dependent transcription for a subset of target genes. Here we identify SIRT2 as a p65 deacetylase. SIRT2 deacetylates p65 in vitro and when overexpressed in cells after TNFα stimulation. Furthermore, endogenous p65 and SIRT2 interact in the cytoplasm of unstimulated cells. Subsequent TNFα treatment does not trigger SIRT2 translocation to the nucleus, but enhances complex formation with p65 after 30 to 60 minutes, thus indicating that it deacetylates p65 in the cytoplasm. Moreover, p65 is hyperacetylated at K310 in *Sirt2*$^{-/-}$ MEFs, which correlates with a strong increase in expression of *Mpa2l*, a gene that is dependent on the acetylation of p65 at K310.

We observed that p65 was rapidly acetylated at K310 in the nucleus of MEFs upon TNFα stimulation and that the signal was drastically reduced after 45 minutes of TNFα treatment (Fig. 1C). Immunostaining and EMSA experiments have previously shown that the majority of NF-κB shuttles back to the cytoplasm between 30 to 60 minutes after TNFα stimulation in MEFs (Buerki et al., 2008; Hoffmann et al., 2002). Strikingly, the timing of p65 deacetylation at K310 correlates with its reported export to the cytoplasm, supporting the idea that p65 is deacetylated in the cytoplasm.

HDAC3 and SIRT1 have been previously described to deacetylate p65 (Chen et al., 2001; Yeung et al., 2004). However, we did not detect p65 deacetylation by overexpressed HDAC3 in HEK293T cells. This discrepancy might be due to different experimental procedures, such as the use of HEK293T cells instead of COS-7 cells. Deacetylation of K310 by SIRT1 was shown in vitro with recombinant proteins and in HEK293T cells with

overexpressed proteins, which correlates with our results; however, the latter might be the consequence of overexpressing the proteins. Hyperacetylation of K310 upon TNFα stimulation in the *Sirt2*$^{-/-}$, but not *Sirt1*$^{-/-}$ MEFs suggests that SIRT2, but not SIRT1, is crucial to deacetylate K310 of p65 in cells. Our experiments with *Sirt1*$^{-/-}$ cells are in agreement with observations where RNAi of SIRT1 did not lead to changes in NF-κB target gene expression, unless cells were treated with resveratrol (Yeung et al., 2004). These results suggest that SIRT1 and SIRT2 might function independently in p65 deacetylation and do not exclude a role for SIRT1 in the deacetylation of other lysines in p65. Recently, a study showed that SIRT6 does not deacetylate p65 in vitro or in vivo, in agreement with our results (Fig. 2B,C), but deacetylates histone H3K9 to attenuate NF-κB signaling (Kawahara et al., 2009).

Gene expression analysis of *Sirt2*$^{-/-}$ MEFs revealed that transcriptional activation of *Mpa2l*, whose expression was dependent on p65 acetylation at K310 (supplementary material Fig. S3B), was dramatically increased in *Sirt2*$^{-/-}$ cells compared with another gene, *Rela*, whose expression does not depend on K310 acetylation (Fig. 4D). This effect was not just restricted to *Mpa2l*. Our extended real-time PCR analysis on 12 other NF-κB target genes revealed six of the tested genes to be more inducible upon TNFα stimulation in *Sirt2*$^{-/-}$ cells compared with *Sirt2*$^{+/+}$ cells (Table 1). Therefore, we propose that SIRT2 regulates the expression of a subset of NF-κB-dependent genes by deacetylating p65 at K310 in the cytoplasm upon relocation of NF-κB to this cellular compartment after the first wave of NF-κB induction. Since NF-κB is known to shuttle between nucleus and cytoplasm in 30–60 minutes in MEFs upon continuous TNFα stimulation (Hoffmann et al., 2002), p65 would be hyperacetylated in *Sirt2*$^{-/-}$ cells after 3 hours of TNFα treatment, which would explain the observed differential *Mpa2l* gene regulation.

Crosstalk between deacetylation at K310 and other post-translational modifications on p65 has to be investigated further. For example, phosphorylation of p65 at S276 was reported to enhance its interaction with its transcriptional coactivator CBP/p300 and with components of the general transcription machinery (Zhong et al., 2002). High-resolution microscopy revealed very recently that S536-phosphorylated p65 predominantly localized in the cytosol (Moreno et al., 2010). It is therefore possible that other post-translational modifications of p65 (e.g. phosphorylation of S536) also regulate the formation of complexes of SIRT2 and p65.

Increased gene response after TNFα stimulation in *Sirt2*$^{-/-}$ MEFs might have physiological relevance. Caspase-3 activity assay revealed that *Sirt2*$^{-/-}$ MEFs are more resistant to TNFα-induced apoptosis. After 20 hours of TNFα stimulation, *Sirt2*$^{-/-}$ MEFs showed less than 0.5% increase in caspase-3 activity, whereas *Sirt2*$^{+/+}$ MEFs displayed a ~30% increase in caspase-3 activity (Fig. 4H). Thus, it could be envisioned that enhanced NF-κB activity in *Sirt2*$^{-/-}$ MEFs protects from TNFα-induced apoptosis and has physiological relevance in NF-κB-mediated survival pathways. We also observed that *Sirt2*$^{-/-}$ MEFs proliferate more slowly than *Sirt2*$^{+/+}$ MEFs (data not shown). This suggests that there are other target transcriptional factors of SIRT2, such as E2F, which has been shown to be important for cell proliferation (Polager et al., 2002).

Despite the identification of p65 acetylation, the exact mechanism by which p65 K310 acetylation regulates the expression remains to be further elucidated. Huang and colleagues reported that the double bromodomain of Brd4 binds to acetylated K310, which enhances transcriptional activation of NF-κB and the expression of a subset of NF-κB inflammatory genes in an acetylated-K310-dependent manner (Huang et al., 2008).

Our data suggest that in unstimulated cells, SIRT2 exists in a complex with p65. Upon TNFα stimulation, p65 translocates to the nucleus, whereas SIRT2 stays in the cytoplasm. Coactivator p300 binds to nuclear p65 and acetylates it at K310, K314 and K315 to fine-tune gene expression. Once the NF-κB response is terminated, p65 shuttles back to the cytoplasm, where SIRT2 deacetylates p65 at K310, thereby resetting the whole NF-κB response. Any p65 that would afterwards translocate to the nucleus again during continuous TNFα stimulation would have to be freshly acetylated by p300 to modulate gene expression, thereby allowing a tight control of NF-κB-dependent transcription.

Together, our results identify SIRT2 as a deacetylase of NF-κB and an important regulator of TNFα-induced NF-κB-dependent gene expression.

Materials and Methods

Tissue culture

Complemented *p65*$^{-/-}$ NIH3T3 mouse embryonic fibroblasts (MEFs) stably expressing p65 wild type or the acetylation-deficient mutants were previously described (Buerki et al., 2008). They were maintained in DMEM supplemented with 10% FCS, 100 U/ml penicillin-streptomycin (Gibco) and non-essential amino acids (Gibco). *Sirt2*$^{-/-}$ and *Sirt2*$^{+/+}$ MEFs were a generous gift from Alejandro Vaquero (IDIBELL, L'Hospital et de Llobregat, Barcelona, Spain). Those cells were originally generated in the lab of Fred Alt, HHMI, Boston, MA (Vaquero et al., 2006). *Sirt1*$^{-/-}$ and *Sirt1*$^{+/+}$ MEFs were kindly provided by F. Alt (Cheng et al., 2003). *Sirt1*$^{-/-}$, *Sirt1*$^{+/+}$, *Sirt2*$^{-/-}$ and *Sirt2*$^{+/+}$ MEFs were kept in DMEM supplemented with 15% FCS, 100 U/ml penicillin-streptomycin (Gibco), non-essential amino acids (Gibco), β-mercaptoethanol (Gibco) and sodium pyruvate (Gibco). HEK293T and HeLa cells were maintained in DMEM supplemented with 10% FCS and 100 U/ml penicillin-streptomycin. THP1 cells were maintained in RPMI medium containing 10% FCS, 100 U/ml penicillin-streptomycin (Gibco), non-essential amino acids (Gibco), β-mercaptoethanol (Gibco) and sodium pyruvate (Gibco).

Plasmids

Plasmids for the mammalian expression of human p65 wild type and mutants K310R, K314/315R and KTR are described elsewhere (Buerki et al., 2008). Mammalian expression plasmids of human SIRT1, SIRT2, SIRT6 and SIRT7 in the pcDNA-DEST40-V5/HIS background were kindly provided by Izumi Horikama, NCI, NIH, Bethesda, MD (Michishita et al., 2005). The enzymatic inactive mutant was generated by site-directed mutagenesis of H187 to Y with pcDNA-DEST40-SIRT2 as template. The introduced mutation was verified by DNA sequencing. Plasmids for the expression of HA-tagged SIRT2 and HA vector control were described elsewhere (Pandithage et al., 2008). The GW-pHA-SIRT2-NLS vector was created by cloning the sequence 5′-GATCCCCAAAGAAGAAGCGAAAGG-TAC into GW-pHA-SIRT2. Site-directed mutagenesis was performed to change the SIRT2 K4 and K12 to alanines, creating the GW-pHA-SIRT2-NLSΔNES vector. The introduced sequence and mutations were verified by DNA sequencing.

Reagents and antibodies

Human tumor necrosis alpha (TNFα), Trichostatin A (TSA), Nicotinamide (Nam) and acetyl-Coenzyme A (acetyl Co-A) were purchased from Sigma. Recombinant mouse TNFα was either purchased from Sigma or generated in our laboratory. Sodium fluoride (NaF) and β-glycerophosphate were obtained from Fluka. The acetyl-specific antibodies for p65 anti-acetyl K310 (ab19870), anti-acetyl K314 (ab18727) and anti-acetyl K315 (19869) were generated by Abcam. The following antibodies were purchased from Santa Cruz Biotechnologies: anti-p65 (sc-372), anti-p300 (sc-585) and anti-SIRT2 (sc-20966 and sc-28298). The anti-SIRT1 antibody was from Millipore (07-131). The anti-Myc antibodies were either purchased from Roche (11-667-149-001) or purified from hybridoma cells. The anti-tubulin antibody was purchased from Sigma (T6199), the anti-His was from Qiagen (34670) and the anti-HA was from Covance (MMS-101P). Caspase-3 activity assay was purchased from Calbiochem (QIA70).

Generation of recombinant proteins

The recombinant proteins were expressed by baculovirus in Sf21 cells using either the FastBac or the BacPAK systems (Clontech). His-tagged proteins were purified over Ni^{2+} beads (ProBond, Invitrogen) and GST-tagged proteins over L-Glutathione beads (Sigma).

In vitro acetylation assay

1 μg of recombinant p65 wild type or the acetylation-deficient mutants were incubated with 500 ng recombinant p300 in HAT buffer (50 mM Tris-HCl, pH 8, 100 mM NaCl, 10% glycerol, 1 mM PMSF, 1 mM DTT, 1 μg/ml bepstatin, 1 μg/ml leupeptin, 1 μg/ml pepstatin and 1 mM sodium butyrate) with or without addition of 150 μM acetyl-CoA. After 1 hour at 30°C, samples were resolved on SDS-PAGE and analyzed by western blot.

In vitro deacetylation assay

500 ng recombinant p65 wild type was in vitro acetylated as described previously and then immunoprecipitated. The bead-bound p65 was incubated with 14 pmol of each recombinant SIRT in 100 μl deacetylation buffer (50 mM Tris-HCl, pH 9, 4 mM $MgCl_2$, 0.2 mM DTT, 1 μg/ml bepstatin, 1 μg/ml leupeptin and 1 μg/ml pepstatin) supplemented or not with 1 mM NAD^+ for 30 minutes or 2 hours with constant agitation. Proteins were subsequently resolved in SDS-PAGE and analyzed by western blot. Purified recombinant SIRTs were additionally resolved on SDS-PAGE and stained with Coomassie Blue.

Acetylation and deacetylation assays in cells

HEK293T cells were transfected with expression plasmids for p300 and either Myc-tagged p65 wild type, the acetylation-deficient mutants or an empty vector, using the calcium phosphate precipitation method. After 23 hours, cells were treated with 10 ng/ml human TNFα for 30 minutes. Then, whole-cell extracts were prepared and 40 μg protein was analyzed by SDS-PAGE and western blot. For the deacetylation assay in cells, 0.1 pmol expression plasmid encoding the different sirtuins (wild type or mutants) or empty vector were additionally co-transfected in HEK293T cells.

Immunostaining

Transfected HEK293T cells or HeLa cells were stimulated with 10 ng/ml human TNFα for 30 minutes. They were fixed with 4% paraformaldehyde and then permeabilized with 0.2% Triton X-100. Blocking solution (2% BSA and 0.1% Triton X-100 in PBS) was added for 1 hour before the slides were incubated with anti-HA and anti-p65 antibodies (1:250 each in blocking solution), followed by incubation with FITC-labeled anti-mouse and Cy3-labeled anti-rabbit antibodies (1:250 each in blocking solution, Jackson ImmunoResearch). The slides were washed, covered with Vectashield mounting solution (Vector Laboratories) and visualized using a Leica SP5 confocal microscope.

Caspase-3 activity assay

Caspase-3 activity assay was performed according to manufacturer's instructions (Calbiochem). Briefly, 7×10^3 cells were seeded on 96-well plates. 5 hours after seeding, cells were stimulated with TNFα (100 ng/ml) or campotethcin (50 μM). After 20 hours of stimulation, cells were centrifuged on the plates and analyzed for caspase-3 activity using microplate Reader Tecan Infinite 200.

Nuclear and cytoplasmic extract preparation and immunoprecipitation

THP1 cells were stimulated with 10 ng/ml TNFα for the indicated time periods, harvested, washed once with PBS, and washed three times in buffer A (10 mM HEPES-KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1 μg/ml pepstatin, 1 μg/ml bestatin, 1 μg/ml leupeptin, 2 mM PMSF, 5 mM DTT). Cells were lysed with A+ buffer (Buffer A+ 0.05% NP-40) and centrifuged for 5 minutes at 5000 r.p.m. Supernatant corresponding to cytoplasm was saved and kept on ice until the nuclear fraction was ready. The pellet was washed three times with buffer A and subsequently resuspended in three volumes of Buffer C (20 mM HEPES-KOH, pH 7.9, 0.42 M NaCl, 1.5 mM $MgCl_2$, 25% v/v glycerol, 1 μg/ml pepstatin, 1 μg/ml bestatin, 1 μg/ml leupeptin, 2 mM PMSF, 5 mM DTT) and incubated for 15 minutes at 4°C on rotating wheels. Lysate was centrifuged for 10 minutes at 4°C at 14,000 r.p.m. and the supernatant corresponding to nuclear extract was separated for immunoprecipitation. 150 μg nuclear extracts or 450 μg cytoplasmic extracts were mixed with IP buffer (20 mM HEPES-KOH, pH 7.9, 2.5 mM $MgCl_2$, 0.1% NP-40, 50 mM NaCl, 1 μg/ml pepstatin, 1 μg/ml bestatin, 1 μg/ml leupeptin, 2 mM PMSF) in a final of 120 mM NaCl concentration, 2.5% of the mix was saved for the input analysis. p65 antibody was added and incubated for 2 hours at 4°C on a roller. At the end of the incubation, prewashed protein-G beads were added to the mix for another 2 hours. Finally, beads were washed three times with IP buffer containing 120 mM NaCl. Beads and the inputs were added Laemmli buffer, boiled and resolved on SDS-PAGE and subjected to western blot using different antibodies.

Gene expression by real-time RT-PCR

Complemented MEFs were starved overnight before treatment with 30 ng/ml recombinant TNFα for different time points. *Sirt2*$^{+/+}$ and *Sirt2*$^{-/-}$ MEFs were starved for 6 hours and then stimulated with 30 ng/ml recombinant TNFα for 3 hours or left unstimulated. Total RNA was isolated from at least two biological samples at different days with the 'Total RNA isolation mini kit' (Agilent Technologies). RNA was subsequently retro-transcribed using the 'High-capacity cDNA reverse transcription kit' (Applied Biosystems). Real-time PCR was performed using the Rotor-Gene 3000 (Corbett Life Science, now Qiagen) and TaqMan assays from Applied Biosystems for *Mpa2l*, *Mmp13*, *Rps6* and *CanX* genes. The last two genes were used as internal controls to normalize for RNA input. In addition, gene expression of *Mpa2l*, *Mmp13*, *Rela* and *Rps12* (as internal control) was assessed with SYBR Green using the primers listed in supplementary material Table S1. RNA from at least two biological replicates per sample was measured and analyzed with REST (Pfaffl et al., 2002). Each experiment was run independently three times, the mean value ± s.d. was calculated and plotted. Expression of genes presented in Table 1 was measured with real-time RT-PCR with SYBR Green primers listed in supplementary material Table S1.

Transient transfections

MEF cells were seeded on six-well tissue culture plates and next day transfected with 30 pmol siRNA oligos (Qiagen) with 4 μl Lipofectamine RNAimax reagent (Invitrogen) according to the manufacturer's protocol for 2 days.

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Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/123/24/4251/DC1

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