Acetylation of p65 at lysine 314 is important for late NF-κB-dependent gene expression

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Abstract

Background: 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. We have earlier reported that p65, a subunit of NF-κB, is acetylated in vitro and in vivo at three different lysines (K310, K314 and K315) by the histone acetyltransferase p300.

Results: In this study, we describe that site-specific mutation of p65 at lysines 314 and 315 enhances gene expression of a subset of NF-κB target genes including Mmp10 and Mmp13. Increased gene expression was mainly observed three hours after TNFα stimulation. Chromatin immunoprecipitation (ChIP) experiments with an antibody raised against acetylated lysine 314 revealed that chromatin-bound p65 is indeed acetylated at lysine 314.

Conclusions: Together, our results establish acetylation of K314 as an important regulatory modification of p65 and subsequently of NF-κB-dependent gene expression.

Background

The inducible transcription factor NF-κB has an important function in regulating immune and inflammatory responses, apoptosis, cell proliferation and differentiation and tumorigenesis [1-3]. NF-κB is induced in almost all cell types by different extracellular stimuli causing the activation of an enormous array of target genes [4]. The NF-κB transcription factor family comprises NFκB1 (p50/p105), NFκB2 (p52/p100), p65 (RelA), c-Rel and RelB, which form homo- and heterodimers. The most abundant, best-studied and “classical” form of NF-κB is a heterodimer consisting of the two subunits p50 and p65. In most unstimulated cells, NF-κB is found as inactive transcription factor complex through its physical association with one of the several inhibitors of NF-κB (IκB) [5]. This family of IκB’s includes IκBα, IκBβ, IκBγ, IκBε (p105/p50, C-terminus), p100/p52 (C-terminus), IκB-R and Bcl-3. Virtually all cell types show NF-κB responses where the activity of NF-κB is specifically regulated at multiple levels [1,3,6]: the level of protein synthesis, the existence of at least 12 different NF-κB dimers, the interaction of these dimers with specific IκBs and their subcellular localization, post-translational modification of these dimers in the cytoplasm and the nucleus, differential accessibility of κB sites in various promoter and enhancer, differential binding to κB’s response elements due to different affinities, and cell type and stimuli specific interaction with a combination of cofactors.

NF-κB is subject to a variety of post-translational modifications (e.g., phosphorylation [7], ubiquitination [8] or prolyl-isomerisation [9]) that modulate its activity. Phosphorylation of the p65 subunit by the PKA, MSK1 and PKCζ kinases enhances its interaction with the co-activator p300/CMBP and stimulates the NF-κB transcriptional activity [7,10-12], while dephosphorylation of p65 by the phosphatase WIP1 negatively affected the interaction with p300 [13]. It has recently been shown that p65 and p50 are reversibly acetylated by p300 and P/CAF [14-16]. Chen et al. identified lysine residues (K) 218, 221 and 310 of p65 as acceptor sites for p300 acetylation. Kiernan et al. identified K122 and 123 in p65 as acetylation sites modified by both p300 and P/CAF. A recent report presented the TGF-β1-mediated acetylation of p65 at K221 in vitro and in vivo enhancing the induced activation of NF-κB by bacteria [17]. We recently confirmed acetylation on K314 and 315, two novel acetylation sites [18]. Genetic
complementation of \( p65 \) knockout (-/-) cells with wild type and acetylation-deficient mutants of \( p65 \) revealed that neither shutting, DNA binding nor the induction of anti-apoptotic genes by TNF\( \alpha \) was affected by acetylation on these residues. Micro array analysis of these cells treated with TNF\( \alpha \) for only 45 minutes identified specific sets of genes differently regulated by wild type or acetylation-deficient mutants of \( p65 \) [18]. Specific genes were either stimulated or repressed by the acetylation-deficient mutants when compared to \( p65 \) wild type. These results support the hypothesis that site-specific p300-mediated acetylation of \( p65 \) regulates the specificity of NF-\( \kappa B \)-dependent gene expression.

Here, we extended the gene expression analysis to three hours after TNF\( \alpha \) stimulation and identified genes, which are higher expressed by mutating K314 and 315. ChIP experiments with antibodies directed against acetylated K314 revealed that this lysine is indeed acetylated when \( p65 \) is bound to chromatin. Together our results provide evidence that acetylation of K314 is important for the regulation of NF-\( \kappa B \)-dependent gene expression in vivo.

**Results**

**Mutation of \( p65 \) K314/315 regulates TNF\( \alpha \)-induced NF-\( \kappa B \)-dependent gene expression at 3 hours**

We provided earlier evidence that acetylation of \( p65 \) at K310, 314 and 315 is important for the expression of a defined subset of genes [18]. These earlier studies provided a first glance of the functional relevance of \( p65 \) acetylation, since gene expression was measured only after 45 minutes of TNF\( \alpha \) stimulation. In order to know if the requirement for site-specific acetylation is maintained for the same genes after longer exposure to TNF\( \alpha \), and to identify possible new genes regulated through \( p65 \) acetylation, we decided to extend our analysis to 3 hours of stimulation. For this, we used \( p65 \) (-/-) mouse embryonic fibroblasts (MEFs) complemented with acetylation-deficient mutants, where the target lysine for acetylation were mutated to arginines. These cells were described and extensively characterized previously [18]. \( p65 \) (-/-) cells complemented either with wild type \( p65 \), an empty plasmid as control (pTV), the acetylation-deficient double mutant K314/315R or the triple mutant KTR (K310/314/315R), were stimulated by TNF\( \alpha \) for 3 hours and total RNA was isolated in three independent replicates from these cells. RNA was amplified, labeled and hybridized to the Agilent Whole Mouse Genome Array. After statistical analysis of the expression profiles, differentially expressed genes were identified (Fig. 1A-B, and Tables 1 and 2). We focused only on genes that required \( p65 \) for their proper induction, which were identified by comparing gene expression profiles from wild type and pTV cells. The majority of differentially expressed genes in KTR mutant were strongly downregulated compared to wild type cells, suggesting that acetylation of \( p65 \) at these residues is also an important modification for the expression of the extended NF-\( \kappa B \)-dependent gene expression (Fig. 1A). In contrast, experiments with the double K314/315R mutant revealed that the majority of genes were slightly upregulated after 3 hours compared to wild type cells (Fig. 1B).

**Gene expression of Mmp10 and Mmp13 is enhanced when K314/315 are mutated**

We subsequently investigated the induction kinetics of several genes by real-time RT-PCR in the complemented cell lines stimulated by TNF\( \alpha \) for different time points (between 20 and 360 minutes). Selection of these genes was based on their inducibility by TNF\( \alpha \), as well as their dependency on \( p65 \) and their regulation by acetylation of K314/315. \( Mmp13 \) and \( Mmp10 \) represented genes that were upregulated in the micro array experiments of K314/315R but not affected in KTR expressing cells. In contrast, \( Cfb \) and \( Mpa2l \) represented genes that were not affected in cells expressing K314/315R, but KTR, suggesting that acetylation of K310 is important for these genes. Overall, the absolute mRNA levels of \( Mmp10 \) and \( Mmp13 \) were strongly and significantly increased upon time (with a maximum value at 6 and 3 hours, respectively) in cells expressing the K314/315R mutant (Fig. 2A, B and Additional file 1: Supplemental Table S1), while the expression levels of \( Cfb \) and \( Mpa2l \) were not affected in the same cells (Fig. 2C, D and Additional file 1: Supplemental Table S1), corroborating our micro array results. Gene expression analysis in cells expressing the KTR mutant indeed confirmed that K310 is important for \( Cfb \) and \( Mpa2l \), but counteracts the effect of K314/315R for \( Mmp10 \) or \( Mmp13 \) expression. Interestingly, the dependency on K310 acetylation for \( Cfb \) and \( Mpa2l \) was already observed when the basal expression levels were analyzed (0 hour time point).

**Characterization of antibodies raised against acetylated lysine 314 and 315 of \( p65 \)**

To further assess the functional relevance of \( p65 \) acetylation in vivo, we generated different antibodies raised against the acetylated K314 or K315. All raised antibodies recognized their specific p300-mediated acetylated residues on recombinant \( p65 \) acetylated in vitro (Fig. 3A) or on over expressed \( p65 \) acetylated in vivo (Fig. 3B).

To confirm that endogenous \( p65 \) is indeed acetylated at the indicated lysines, immunoprecipitation experiments with the antibodies raised against acetylated K314, K315 or against \( p65 \) were performed. The antibody anti-acK314 immunoprecipitated \( p65 \) only in cells complemented with wild type \( p65 \) in a TNF\( \alpha \)-dependent manner (Fig. 3C). No \( p65 \) was immunoprecipitated in
Table 1 Down- and upregulated genes in KTR cell line compared to wild type control

| RefSeq RNA | Gene symbol | Gene name                                       | Fold change | P-value   |
|------------|-------------|------------------------------------------------|-------------|-----------|
| XR_001627  | A630026L20  | Hypothetical protein A630026L20                | 0.188       | 9.40E-30  |
| NM_029000  | Gvin1       | GTPase, very large interferon inducible 1      | 0.26        | 8.04E-09  |
| NM_024435  | Nts         | Neurotensin                                    | 0.262       | 5.50E-10  |
| NM_133871  | If44        | Interferon-induced protein 44                  | 0.282       | 0.001     |
| NM_146015  | Efemp1      | Epidermal growth factor-containing fibulin-like extracellular matrix protein 1 | 0.286 | 2.31E-20 |
| NM_145153  | Oas1f       | 2'-5' oligoadenylate synthetase 1F             | 0.349       | 0.000045  |
| NM_199015  | D14Ertd668e | DNA segment, Chr 14, ERATO Doi 668, expressed  | 0.368       | 0.000005  |
| NM_145211  | Oas1a       | 2'-5' oligoadenylate synthetase 1A             | 0.369       | 0.001     |
| NM_172603  | Phf11       | PHD finger protein 11                          | 0.371       | 3.08E-07  |
| NM_009099  | Trim30      | Tripartite motif protein 30                    | 0.378       | 0.001     |
| NM_183249  | 1100001G20Rik | RIKEN cDNA 1100001G20 gene                   | 0.395       | 8.10E-07  |
| NM_030150  | Dhex58      | DEXH (Asp-Glu-X-His) box polypeptide 58       | 0.413       | 0.000006  |
| NM_021394  | Zbp1        | Z-DNA binding protein 1                        | 0.419       | 0.000443  |
| NM_088690  | Nrkbie      | Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon | 0.422 | 0.000005 |
| XM_001000862 | I830012O16Rik | RIKEN cDNA I830012O16 gene                   | 0.422       | 0.002     |
| NM_021792  | Iigg1       | Interferon inducible GTPase 1                  | 0.427       | 0.000001  |
| NM_194336  | Mpa2i       | Macrophage activation 2 like                   | 0.435       | 0.039     |
| NM_007969  | Exp1        | Extracellular proteinase inhibitor             | 0.436       | 0.000025  |
| NM_029000  | Gvin1       | GTPase, very large interferon inducible 1      | 0.438       | 0.000024  |
| NM_009099  | Trim30      | Tripartite motif protein 30                    | 0.454       | 0.000002  |
| NM_011109  | Usp18       | Ubiquitin specific peptidase 18                 | 0.458       | 0.002     |
| NM_029803  | If272a      | Interferon, alpha-inducible protein 27 like 2A | 0.46        | 0.000163  |
| NM_029803  | If272a      | Interferon, alpha-inducible protein 27 like 2A | 0.462       | 0.000471  |
| NM_088200  | H2-D4       | Histocompatibility 2, D region locus 4         | 0.483       | 0.000006  |
| NM_010501  | Ifi3        | Interferon-induced protein with tetraticopeptide repeats 3 | 0.488 | 0.000741 |
| NM_153564  | Gbp5        | Guanylate binding protein 5                    | 0.493       | 0.000843  |
| NM_013606  | Mx2         | Myxovirus (influenza virus) resistance 2       | 0.509       | 0.000002  |
| NM_198095  | Bst2        | Bone marrow stromal cell antigen 2             | 0.511       | 0.000046  |
| NM_009318  | Tapbp       | TAP binding protein                            | 0.511       | 0.004     |
| NM_172777  | BC057170    | cDNA sequence BC057170                        | 0.519       | 0.000028  |
| NM_018734  | Gbp3        | Guanylate binding protein 3                    | 0.52        | 0.000867  |
| NM_001001892 | H2-K1    | Histocompatibility 2, K1, K region             | 0.522       | 7.26E-07  |
| NM_011579  | Tgtp        | T-cell specific GTPase                        | 0.522       | 0.000163  |
| NM_008198  | Cfb1        | Complement factor B                            | 0.523       | 0.000011  |
| NM_010395  | H2-T10      | Histocompatibility 2, D region locus 1         | 0.527       | 0.000001  |
| NM_007936  | Epha4       | Eph receptor A4                                | 0.528       | 0.001     |
| NM_010545  | Cd74        | CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated) | 0.532 | 2.94E-09 |
| NM_008331  | Ifi1        | Interferon-induced protein with tetraticopeptide repeats 1 | 0.532 | 0.002     |
| NM_001001892 | H2-K1    | Histocompatibility 2, K1, K region             | 0.533       | 5.43E-08  |
| NM_028749  | Npl         | N-acetylneuraminate pyruvate lyase              | 0.533       | 0.000004  |
| NM_010380  | H2-D1       | Histocompatibility 2, D region locus 1         | 0.533       | 0.000007  |
| NM_015783  | Igf15       | ISG15 ubiquitin-like modifier                  | 0.534       | 0.008     |
| NM_173743  | 2310016F22Rik | RIKEN cDNA 2310016F22 gene                 | 0.535       | 0.000166  |
| NM_011314  | Saa2        | Serum amyloid A 2                              | 0.536       | 3.53E-07  |
| NM_008330  | If47        | Interferon gamma inducible protein 47          | 0.542       | 0.000013  |
| NM_172826  | Daat2       | Dapper homolog 2, antagonist of beta-catenin (xenopus) | 0.544 | 0.008     |
| NM_021384  | Rsd2        | Radical S-adenosyl methionine domain containing 2 | 0.547 | 0.005     |

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cells expressing the K314/315R or the KTR p65 mutant (Fig. 3D), suggesting that endogenous p65 is indeed acetylated at K314 upon stimulation with TNFα. Unfortunately, anti-acK315 was not specific enough to recognize endogenous acetylation at K315.

Chromatin-associated p65 is acetylated at lysine 314

From the above selected genes only Cfb was already described to contain a κB site in its promoter [19]. We therefore searched for putative κB sites within the DNA sequence 1 kB upstream of the transcription start site (TSS) of the selected genes. Bioinformatic analysis identified several putative κB sites in the promoters of Mmp10 and Cfb, one site in the promoter of Mpa2l, but none for Mmp13 (Fig. 4A). Mmp13 was thus not further investigated. ChIP studies in p65(+/+) MEFs stimulated with TNFα for 20 and 180 minutes revealed that p65 was recruited to the promoters of Cfb and Mpa2l in a stimulus-dependent manner, while no enrichment was observed in p65(−/−) MEFs (Fig. 4B). These recruitments were promoter-specific, since p65 occupancy to promoter of glucagon, a negative control, was not induced upon TNFα stimulation. IP-10, a known NF-κB target gene with very well characterized κB sites at its promoter, served additionally as a positive control. Unfortunately, no p65 enrichment could be observed to the κB sites of Mmp10 (data not shown), indicating that p65 would activate this gene through other κB sites or other transcription factors.

| RefSeq RNA  | Gene symbol | Gene name                                      | Fold change | P-value     |
|-------------|-------------|------------------------------------------------|-------------|-------------|
| NM_007377   | Aatk        | Apoptosis-associated tyrosine kinase           | 2.995       | 7.21E-29    |
| NM_009876   | Cdkn1c      | Cyclin-dependent kinase inhibitor 1C (P57)     | 2.979       | 1.99E-09    |
| NM_172119   | Ddx3        | Deidinase, iodothyronine type III              | 2.685       | 2.31E-07    |
| NM_027406   | Aldh1l1     | Aldehyde dehydrogenase 1 family, member L1    | 2.39        | 1.35E-09    |
| NM_008342   | Igrp2       | Insulin-like growth factor binding protein 2   | 2.381       | 0.000196    |
| NM_010942   | Nsg1        | Neuron specific gene family member 1           | 2.254       | 0.000001    |
| NM_008607   | Mmp13       | Matrix metallopeptidase 13                     | 2.222       | 4.57E-07    |
| NM_010942   | Nsg1        | Neuron specific gene family member 1           | 2.119       | 0.000013    |
| NM_028072   | Sulf2       | Sulfatase 2                                    | 2.1         | 0.000004    |
| NM_027251   | 2010107G23Rik | RIKEN cDNA 2010107G23 gene                   | 2.057       | 1.81E-07    |
| NM_019955   | Ripk3       | Receptor-interacting serine-threonine kinase 3 | 2.047       | 5.15E-10    |
| NM_133888   | Smad3b      | Sphingomyelin phosphodiesterase, acid-like 3B  | 2.04        | 8.17E-19    |
| NM_001081421| Galtnt1     | UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylglactosaminyltransferase-like 1 | 2.005 | 0.000414 |
| NM_028072   | Sulf2       | Sulfatase 2                                    | 1.956       | 1.26E-07    |
| NM_199252   | Unc93a      | Unc-93 homolog A (C. elegans)                  | 1.938       | 0.000978    |
| XM_283765   | S40433G21Rik | RIKEN cDNA S40433G21 gene                   | 1.915       | 0.00025     |
| NM_080563   | Rnf144      | Ring finger protein 144                       | 1.83        | 0.006       |
| NM_019471   | Mmp10       | Matrix metallopeptidase 10                    | 1.829       | 0.00263     |
| NM_009971   | Csf3        | Colony stimulating factor 3 (granulocyte)      | 1.807       | 0.000516    |
| NM_029000   | Gwnt1       | GTPase, very large interferon inducible 1      | 0.501       | 0.005       |
| NM_009099   | Trimm30     | Tripartite motif protein 30                   | 0.506       | 0.036       |
| AX077243    | I830012016Rik | RIKEN cDNA I830012016 gene                  | 0.509       | 0.008       |
| NM_009606   | Acta1       | Actin, alpha 1, skeletal muscle               | 0.511       | 4.8E-14     |
| NM_153564   | Gbp5        | Guanylate binding protein 5                   | 0.521       | 0.000007    |
| NM_028872   | 573059C18Rik | RIKEN cDNA 573059C18 gene                   | 0.523       | 8.43E-07    |

List of differentially regulated genes in K314/315R versus wild type control at 3 hours TNFα stimulation analyzed by microarrays. Average values from at least two biological replicates are shown.
Figure 1 Site-specific acetylation of p65 regulates the expression of distinct genes. Heat maps showing the gene expression profiles of KTR cells (A) or K314/315R cells (B) compared to wild type control at 3 hours after TNFα stimulation using whole mouse genome arrays. Each row represents a single gene, and each column represents a different cell line. Red or green represents up- or downregulation of genes relative to the mean on each row, respectively. Mean data from at least two biological replicates is displayed. Only genes upregulated in wild type cells compared to pTV control were taken into account (>2-fold, p-value < 0.05). From these genes, only the ones with significant changes in expression levels (>1.8-fold or <0.556-fold, p-value < 0.05) between wild type and mutant cells are shown.

Figure 2 Confirmation of gene expression profiles by qRT-PCR. Gene induction of Mmp10 (A), Mmp13 (B), Cfb (C) and Mpa2l (D) in a TNFα-dependent manner, as measured by real-time RT-PCR, from the following complemented cell lines: wild type (green), KTR (red), K314/315R (blue) and pTV (grey). Samples were normalized to Rps6 and CanX expression levels, and expressed as fold increase relative to wild type unstimulated. Two biological replicates were included. Shown are the means ± SD of three independent runs.
Interestingly, ChIP experiments using the anti-acetyl K314 antibodies showed that chromatin-associated p65 is indeed acetylated at K314 on all analyzed genes (Cfb, Mpa2l and IP-10) (Fig. 4C), although the expression of these genes was not affected by mutating K314/315 (Fig. 2C and 2D). Furthermore, the detected amount of recruited p65, which was acetylated at K314, increased over time (comparing values at 180 min and 20 min). Unfortunately, the antibody raised against acetyl K315 was not able to immunoprecipitate p65 under the tested conditions (data not shown). Together, these experiments identified Mpa2l as a novel NF-κB target gene through the recruitment of p65 to its promoter and provide strong evidence that chromatin-bound p65 is indeed acetylated at K314.

Discussion

We have previously shown that p65 acetylation plays an important role in regulating NF-κB-dependent transcription of a subset of genes. Our current results confirm those earlier findings by identifying new genes differentially regulated in the acetylation-deficient mutants compared to wild type cells. Remarkably, only a few genes identified after 3 hours TNFα stimulation overlapped with the genes identified in our previous study after 45 minutes TNFα stimulation (data not shown). We also observed that TNFα does not induce NF-κB activity in the complemented MEFs as efficiently as in the parental p65(+/+) MEFs used for ChIP (data not shown). Therefore, although our complemented MEFs are useful to address the role of p65 acetylation in vivo, we might have identified only part of the genes influenced by p65 acetylation with the microarray screen.

Cfb was previously suggested to be a direct target of NF-κB by EMSA experiments [19]. Our ChIP assays confirmed that p65 is recruited to Cfb promoter in a TNFα-dependent manner in vivo and further identified Mpa2l as a novel NF-κB target gene. Although Mmp10 and Mmp13 were differentially expressed in acetylation-deficient mutant cells compared to wild type cells, we failed to detect p65 recruitment to those promoters. One explanation could be that p65 is recruited to the promoter region of these genes with a different kinetics than the one we investigated here. Alternatively, p65 could bind to a regulatory element located far away from the promoter to regulate transcription, as has been
shown for several NF-κB target genes [20-22]. NF-κB is known to activate the expression of many transcription factors and their regulators [4]; consequently, a third possibility is that p65 directly induces the expression of a protein that regulates the expression of these genes. Along this line, NF-κB has been shown to directly activate the expression of the transcription factor Elk-1, which in turn induces Mmp13 gene expression [23].

Interestingly, the stimulatory effect of mutating K314/315 was lost when additionally mutating K310, suggesting that K310 acetylation is not affected by the lack of K314/315 acetylation, and that the modification of K310 would counteract the effect of K314/315 at the particular tested genes. Furthermore, the effect of mutating K314/315 on gene expression was more obvious after 3 hours of stimulation compared to early time points (e.g. 20 minutes), suggesting that acetylation of these lysines might more likely affect termination of gene expression than its induction itself. Here, we provide for the first time evidence that endogenous,

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**Figure 4** Acetylated p65 is recruited to the promoter region of regulated genes upon TNFα stimulation (A) Promoters of Cfb, Mpa2l and Mmp10 have putative κB sites. Schematic representation of putative κB sites found in the indicated promoter regions. The distance in bp relative to the transcription start site (TSS) of every putative κB site is shown, as well as the sequence from the κB site chosen for analysis by ChIP. Chromatin immunoprecipitation analysis of p65 (B) or p65acetylated at K314 (C) from p65(+/-) MEFs kept unstimulated or treated with TNFα for 20 or 180 minutes. Chromatin from p65(-/-) MEFs stimulated with TNFα for 180 minutes was used as negative control. Recruitment to the indicated promoters was analyzed. Occupancy to IP-10 and Glucagon promoters was assessed as positive and negative control, respectively. Samples were normalized to input chromatin and expressed as % input. The result is representative of three independent experiments. Mean values ± SD of three independent runs are shown.
chromatin-bound p65 is indeed acetylated at K314 upon stimulation with TNFα. Unfortunately, acetylation of K315 could not be confirmed due to the lack of a specific acetylation-dependent antibody that recognizes endogenous proteins. Interestingly, we observed acetylation of p65 K314 at promoters of genes whose expression was not affected by mutating K314/315 (e.g. IP-10). Thus, it could well be that p65 is acetylated upon recruitment of p300 at promoters of many genes, but that the expression of only some of these genes is affected by p65 acetylation at distinct lysines.

The observation that mutation of K314/315 increases expression of certain genes (e.g. Mmp10 and Mmp13) suggests that acetylation of K314 and possibly K315 might represses gene expression. A possible molecular mechanism is that acetylated lysines at p65 create docking sites for bromodomain-containing proteins which need to be recruited to promoters to modulate transcription [24]. On the other hand, we can currently not exclude that lysines 314 and/or 315 are modified by another post-translational modification (e.g. methylation, [25]) that mutating these residues would thus affect gene expression independently of acetylation. However, provided the observed acetylation of chromatin-bound p65 at K314, acetylation is more likely to compete with another lysine modification, possibly resulting in cross-talk between different modifications [25]. Indeed, a recent study reported that the same two lysines (K314/315) can be methylated by Set9 to induce ubiquitylation of NF-κB and subsequently terminate gene expression [26]. Thus, acetylation at K314 and possibly K315 could prevent methylation-mediated repression of target genes and thus avoid methylation-induced termination of p65-dependent gene expression. A direct evidence for methylated p65 at K314/315 bound to chromatin is however still missing. Whether the same genes are regulated by both post-translational modifications should be further addressed. Alternatively, both post-translational modifications might regulate distinct set of NF-κB-dependent genes and thus not influence each other.

Conclusions
Together, our results establish acetylation of K314 as an important regulatory modification of p65 and subsequently of NF-κB-dependent gene expression.

Methods

Tissue culture
Complemented p65(-/-) NIH 3T3 MEFs stably expressing p65 wild type or the acetylation-deficient mutants were generated by lentiviral complementation as previously described in [18]. Briefly, HEK 293T cells were transfected with the packaging plasmid, the envelope plasmid and pTV-myc-RelA/p65 wild type, RelA/p65 K/R mutants or the control pTV vector. p65(-/-) MEFs were infected with the viral supernatant and split into selective medium (2.5 μg/ml Blasticidin) after thirty-six hours. Expression of recombinant proteins was confirmed by western blot analysis. Pools of cells were used for further analysis. They, as well as the p65(-/-) and (+/+ ) parental NIH 3T3 MEFs kindly provided by A. Beg [27], were maintained in DMEM supplemented with 10% FCS, 100 units/ml penicillin/streptomycin (Gibco) and non-essential amino acids (Gibco). HEK 293T cells were maintained in DMEM supplemented with 10% FCS and 100 units/ml penicillin/streptomycin.

Plasmids
Plasmids for the mammalian overexpression of human p65 wild type and mutants K310R, K314/315R and KTR in HEK 293T cells were described elsewhere [18]. Briefly, ppp-CMV-Km-RelA/p65 wild type, previously described in [28], was used as template vector for the generation of the p65 mutants by site-directed mutagenesis according to the QuickChange protocol (Stratagene). All introduced mutations were confirmed by sequencing.

Reagents and antibodies
Human 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 beta-glycerophosphate were obtained from Fluka. The acetyl-specific antibodies for p65 anti-acetyl K314 (ab18727) and anti-acetyl K315 (ab19869) were generated by Abcam. The following antibodies were purchased from Santa Cruz Biotechnologies: anti-p65 (sc-372) and anti-p300 (sc-585). The anti-myc antibodies were either purchased from Roche (11-667-149-001) or purified from hybridoma cells.

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 Ni2+-beads (ProBond, Invitrogen).

Micro array
Complemented cell lines p65 wild type or acetylation-deficient mutants were starved overnight before either left untreated or stimulated with 30 ng/ml TNFα for 3 hours. Total RNA from three biological replicates per sample was isolated at different days using the ‘Total RNA isolation mini kit’ (Agilent Technologies). RNA quality was measured on the 2100 Bioanalyzer (Agilent Technologies). Microarray experiments were performed using ‘Whole Mouse Genome (4 x 44 K) Oligo Microarray Kit’ (Agilent Technologies) and ‘One-color micro array-based gene expression analysis’ (Agilent Technologies).
Technologies) following the manufacturer’s protocol. Cy3-labeled cRNA was purified with the RNeasy kit (Qiagen). Dye incorporation was assessed with the ND-1000 Spectrophotometer (NanoDrop Technologies). Per sample, 1.65 μg cRNA from each of the three biological replicates was hybridized to independent arrays according to the manufacturer’s protocol. Hybridized slides were scanned with the Agilent DNA Microarray scanner and quantified using the Agilent Feature Extraction software. The data analysis was performed using ‘Rosetta Resolver’ Gene Expression Data Management and Analysis System’ (Rosetta Biosoftware). Briefly, data was processed and normalized with default settings. Then, low-signal genes with signal intensities < 0.1 were filtered out. Differential expression between two conditions was assessed based on the average ratio and significance. All genes with expression ratios < 0.556 and >1.8, and a p-value < 0.05 were selected to generate the tables of significantly regulated genes. These sequence data have been submitted to the GEO database http://www.ncbi.nlm.nih.gov/geo under accession number GSE15196.

**Gene expression by quantitative RT-PCR**

Complemented MEFs were starved overnight before treatment with 30 ng/ml recombinant TNFα for different time points. Total RNA was isolated from at least two biological samples at different days with the ‘Total RNA isolation mini kit’ (Agilent Technologies). RNA quantity was assessed with the ND-1000 Spectrophotometer (NanoDrop Technologies). 2 μg RNA was subsequently retro-transcribed using the ‘High-capacity cDNA reverse transcription kit’ (Applied Biosystems) following the manufacturer’s protocol. Real-time PCR was performed using the Rotor-Gene 3000 (Corbett Life Science, now Qiagen) and TaqMan assays from Applied Biosystems for the following genes: Cfb, Mpa2l, Mmp10, Mmp13, Rps6 and CanX, according to Applied Biosystems’ protocol. The last two genes were used as internal controls to normalize for RNA input. RNA from at least two biological replicates per sample was measured and analyzed by REST [29]. Each experiment was run three independent times, the mean value and ± SD was calculated and blotted into graphs. A 2-tails, paired t-Test was performed with the log of the values to additionally calculate the p-values.

**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 pH8, 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.

**Acetylation assays in cells**

HEK 293T 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.

**Immunoprecipitation**

Whole cell extracts from the complemented cells untreated or stimulated with 10 ng/ml TNFα for 40 minutes were used to immunoprecipitate p65. 750 μg of extract were incubated with 1.5 μg of antibody for 1 hour at 4°C in Co-IP buffer (20 mM HEPES pH 7.9, 100 mM NaCl, 2.5 mM MgCl₂, 0.05% NP-40, 1 mM PMSF, 1 μg/ml pepstatin, 1 μg/ml bestatin, 1 μg/ml leupeptin, 1 μM TSA, 5 mM Nam). Protein G sepharose was added and samples were incubated for another 2 h at 4°C. Samples were washed three times 5 minutes in Co-IP buffer containing 100 mM NaCl before being subjected to 10% SDS-PAGE, followed by western blot.

**Chromatin Immunoprecipitation**

p65(-/-) or (+/+ ) MEFs were stimulated with 10 ng/ml mouse TNFα for the indicated time points and fixed with 1% formaldehyde (Calbiochem) for 10 minutes. After extensive washing, the plasma membrane was first lysed with lysis buffer 1 (50 mM Tris HCl pH8, 2 mM EDTA pH8, 0.1% NP-40, 10% glycerol, 1 mM PMSF, 0.5 mM DTT, phosphatase and HDAC inhibitors) and then the nuclear membrane with lysis buffer 2 (50 mM Tris HCl pH8, 5 mM EDTA pH8, 1% SDS, 1 mM PMSF, 0.5 mM DTT, phosphatase and HDAC inhibitors). Chromatin fragmentation was achieved with the Bioruptor (Diagenode). Sonified chromatin was diluted with 9 volumes of dilution buffer (50 mM Tris HCl pH8, 5 mM EDTA pH8, 0.5% NP-40, 200 mM NaCl and 1 mM PMSF) and pre-cleared for 1 hour with Protein A Agarose/salmon sperm DNA (Millipore). 1% of input was saved and the remaining chromatin was then incubated overnight with the specific antibodies. After 30 additional minutes of incubation with Protein A Agarose/salmon sperm DNA, the immuno-complexes were extensively washed with washing buffer (20 mM Tris HCl pH8, 2 mM EDTA pH8, 1% NP-40, 0.1% SDS, 500 mM NaCl and 1 mM PMSF) and then with buffer TE (10 mM Tris HCl pH8 and 1 mM EDTA pH8). Chromatin was eluted with 2% SDS in TE buffer and incubated at 65°C for at least 6 hours. DNA was purified with ‘QIAquick PCR purification kit’ (Qiagen) following the manufacturer’s recommendations and measured by real-time PCR using SYBR Green and the Rotor-Gene 3000 (Corbett Life Science, now Qiagen). The following primers were used: Mpa2l_forward...
(CAGCCTCCTTTATAGTGCTC), Mpa2l_reverse (TAC AAAATCCGGGAAGTATTGC), Cfb_forward (CACCTTGAGAAGAATGCCTTCT), Cfb_reverse (TTTGTGCAGCAAGGACTCTGACCT), IP-10_forward (GCAATGCCCT CGGGTTTACAG), IP-10_reverse (TTTGTGCAGCAAGGACTCTGACCT), Glucagon_forward (GAGTGGGCGAGTGAAATCAT) and Glucagon_reverse (GCAATGCCCT CGGGTTTACAG). Samples were normalized to input chromatin and expressed as % input. Each experiment was independently repeated at least three times. Mean values ± SD of three independent ChIP runs from one independent ChIP experiment are shown.

List of abbreviations
acetyl Co-AL: acetyl-CoenzymeA; ChIP: chromatin immunoprecipitation; K: lysine; MEFs: mouse embryonic fibroblasts; Nam: Nicotinamide; NF-kB: nuclear factor kB; TNFα: tumor necrosis alpha; TSA: trichostatin A; TSS: transcription start site.

Additional file 1: Gene expression analysis of Mmp10, Mmp13, Cfb and Mpa2l with quantitative RT-PCR. The mean value of three independent runs relative to wild type unstimulated is shown, as well as ± SD and p-values. P-values were calculated comparing data of each cell line with wild type.

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Authors’ contributions
K.M.R. carried out the molecular studies and drafted the manuscript. M.F. participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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