

Threonine phosphorylation of I κ B ζ mediates inhibition of pro-inflammatory target genes

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Abstract

The skin represents the first line of defense against pathogens. Keratinocytes do not only provide an important barrier function, but also act as sensors detecting bacterial, fungal or viral infections. Transcription factors of the NF- κ B family play a crucial role in this process, because they activate the expression of chemokines, cytokines and anti-microbial peptides important for the clearance of the pathogens. In the present study, we discovered that the atypical I κ B member I κ B ζ is strongly induced in keratinocytes sensing the fungal derived glucan zymosan A and that I κ B ζ is essential for the optimal expression of pro-inflammatory genes, such as *IL6*, *CXCL5*, *IL1B* or *S100A9*. So far, it was unclear if I κ B ζ is solely regulated on the transcriptional level. Using mass spectrometry analyses, we identified several I κ B ζ phosphorylation sites, including a cluster of threonine residues located in the N-terminus of the protein, which can be phosphorylated by MAPKs. Surprisingly, I κ B ζ phosphorylation at this threonine cluster promotes the recruitment of HDAC1 to target gene promoters and thus controls the termination of transcription. Taken together, we propose a novel model how an anti-fungal response translates to the expression of pro-inflammatory cytokines and highlight a new layer of complexity in the regulation of the NF- κ B response in keratinocytes.

Keywords: I κ B ζ , NF κ B, NF- κ B, HDAC, inflammation

Introduction

Recognition of pathogen-associated molecular patterns is critical for an efficient innate immune response against pathogens. β -1,3-glucan, a component of bacterial and fungal cell walls, is recognized by the C-type lectin receptor Dectin-1 or Toll-like receptor (TLR)-2 [1]. Receptor engagement by β -glucans, such as zymosan A, on keratinocytes or other cell types elicits the secretion of several pro-inflammatory cytokines, chemokines and anti-microbial peptides, which subsequently activate dendritic cells and T helper cells thus triggering an efficient antimicrobial defense [2, 3].

NF- κ B, which constitutes a family of transcriptional activators that modulate the expression of various genes involved in cell survival and inflammation, is a crucial regulator of innate immune responses against pathogens [4]. The five members of the NF- κ B family, p65 (RelA), RelB, c-Rel, p105/p50 and p100/p52, associate with each other to form various homo- and hetero-dimeric complexes. All NF- κ B proteins share the Rel homology region that is critical for their DNA binding, dimerization and nuclear localization. The proteins p65, RelB and c-Rel have the ability to activate transcription via a C-terminal transactivation domain, which is absent in p50 and p52. Since various stimuli activate the NF- κ B pathway and a great diversity of genes is regulated by NF- κ B, a precise control of its activity is required to avoid misguided cellular responses. The classical activation of NF- κ B is controlled by cytoplasmic inhibitory proteins, such as I κ B α , which sequester NF- κ B in the cytoplasm. Inflammatory stimulation of cells results in the rapid activation of I κ B kinase (IKK), which triggers the phosphorylation-induced degradation of I κ B α , leading to NF- κ B's nuclear translocation and transcriptional induction of target genes [5].

Recent evidence reveals that the activation of NF- κ B target genes is far more complex and dependent on the particular gene context or stimulus, which is thought to facilitate a selective gene regulation in distinct physiological settings. Protein kinases are not only required for phosphorylation of the classical cytoplasmic I κ B proteins leading to their proteasomal degradation, but also affect the transcriptional activity of NF- κ B proteins themselves [5]. For instance, p65 phosphorylation by PKA has been found to displace inhibitory histone deacetylase 1 (HDAC 1) corepressor complexes from target genes and to promote the interaction of p65 with activating histone acetyltransferases [6].

In addition to posttranslational modifications including ubiquitination, acetylation and phosphorylation, so-called atypical I κ B proteins have been identified as an important new control layer regulating NF- κ B target gene expression. Similar to classical I κ B proteins, atypical I κ B proteins, including I κ B ζ , BCL-3, I κ B_{NS} and I κ B η , contain several ankyrin repeats that are normally responsible for NF- κ B inhibition [7]. However, atypical proteins are distinguished by a number of molecular features. They are not constitutively expressed in the cytoplasm but

can be induced by pro-inflammatory stimuli and localize to the nucleus. Most importantly, several atypical I κ B proteins cannot only inhibit, but also induce a particular subset of NF- κ B target genes. The mechanisms of this differential gene regulation by I κ B ζ remains largely unknown, but increasing evidence suggests that the transcriptional activity of I κ B ζ is mainly mediated at the level of chromatin remodeling [8].

In keratinocytes, the expression of I κ B ζ is induced by pro-inflammatory cytokines such as IL-17 in combination with TNF α or IL-1-family cytokines (e.g. IL-1 β , IL-36) [9, 10]. The cytokine-mediated I κ B ζ induction is essential for the expression of important cytokines (e.g. IL-6, IL-17C), chemokines (e.g. CXCL5, CCL2) and antimicrobial peptides (e.g. S100A7, DEFB4A), which collectively mount an efficient antimicrobial host response but also drive pathological skin inflammation such as psoriasis. Intriguingly, I κ B ζ knockout mice are completely resistant to psoriasis induction and psoriasis patients exhibit high I κ B ζ levels in the skin, underlining I κ B ζ central role in the regulation of skin inflammation [9, 10].

How the activity of the I κ B ζ protein is regulated, is almost completely unknown. In addition to ankyrin repeats, I κ B ζ contains a putative transactivation domain, but lacks DNA-binding activity, which is rather conferred through its interaction with the NF- κ B members p50 or p52 [7]. So far, a modulation of I κ B ζ activity by posttranslational modifications has not yet been described. In the present study, we show that zymosan A is a potent inducer of I κ B ζ expression in keratinocytes, that it is important for the expression of pro-inflammatory genes and that it is phosphorylated at multiple site in the N-terminus. Additionally, we demonstrate that a conserved cluster of three adjacent threonine residues of I κ B ζ can be phosphorylated by the Ras/MAPK signaling pathway and that this event does not alter the intrinsic transactivation activity of I κ B ζ . Instead, threonine phosphorylation imposes a negative regulatory signal on I κ B ζ by resulting in an increased recruitment of the co-repressor HDAC1. As HDAC1 attenuates the expression of selected I κ B ζ target genes, our results suggest that I κ B ζ phosphorylation provides a negative feed-back mechanism that terminates the transcription of a specific subset of inflammatory genes.

Results and Discussion

Zymosan stimulation of keratinocytes induces I κ B ζ expression

We recently demonstrated that keratinocytes are able to detect fungal infections by engagement of the C-type lectin receptor Dectin-1 and Toll-like receptor 2 (TLR2), which are able to recognize the yeast-derived β -glucan zymosan A (ZymA) [2]. To investigate whether ZymA induces the expression of I κ B ζ , we treated human primary keratinocytes either with ZymA or with pro-inflammatory cytokines such as IL-17A, TNF α , IL-1 β , with living bacteria,

such as *S. aureus* and *E. coli*, or with bacterial peptidoglycans (PGN) and compared the I κ B ζ protein levels by Western blot. We noticed that ZymA was one of the strongest inducers of I κ B ζ expression in keratinocytes (**Fig 1A**). As described for IL-17A/TNF α and IL-36-mediated I κ B ζ induction [9, 10], expression of I κ B ζ upon ZymA treatment of human primary keratinocytes or HaCaT cells was rapid but transient (**Fig 1B**). Since also other human skin-derived cell lines, such as KYSE-150, A431, BIRC56, SCC-9 and SCC-25, exhibit I κ B ζ induction, we concluded that I κ B ζ upregulation is a common feature of skin cells upon ZymA stimulation (**Fig EV1A**). Even though I κ B ζ is discussed as a primary NF- κ B target gene, sole activation of NF- κ B is not sufficient to drive I κ B ζ expression. This is for example reflected by the fact that TNF α treatment, which triggers canonical NF- κ B activation, is unable to induce I κ B ζ protein expression (**Fig 1A**) [9, 10]. One explanation might be that optimal I κ B ζ protein expression additionally requires the stabilization of *NFKBIZ* transcripts encoding I κ B ζ [11-14]. For example, the nucleases regnase-1 and roquin, which negatively regulate *NFKBIZ* transcript stability, can be inactivated by the protease MALT1 in activated T cells [13]. Indeed, we could recently show that MALT1 becomes activated and cleaves regnase-1 in ZymA-stimulated keratinocytes, thus suggesting that I κ B ζ is induced by ZymA by both NF- κ B activation and MALT1-mediated *NFKBIZ* mRNA stabilization [2].

In IL-17/TNF α - or IL-36-stimulated keratinocytes, I κ B ζ expression is essential for the induction of central immune modulators such IL-6, S100A7 or IL-20 [9, 10]. To test whether I κ B ζ is important for ZymA-induced gene expression, we measured the mRNA levels of multiple cytokines and chemokines in I κ B ζ -silenced human primary keratinocytes or HaCaT cells. Several pro-inflammatory molecules, such as *IL6*, *IL1B* and *S100A7*, were induced by ZymA stimulation, but were strongly reduced in their expression by an I κ B ζ -specific shRNA (**Fig 1C** and **1D**). To further demonstrate the central role of I κ B ζ in the induction of pro-inflammatory genes, we generated I κ B ζ knockout HaCaT cells using CRISPR/Cas9 and reconstituted them with a doxycycline-regulated construct coding for FLAG-I κ B ζ (Tet-On), allowing induction of I κ B ζ expression independent of ZymA stimulation (**Fig 1E** and **Fig EV1B**). Interestingly, I κ B ζ expression alone was sufficient to induce the expression of several pro-inflammatory genes encoding cytokines (e.g. *IL6*, *IL20*), chemokines (e.g. *CXCL5*, *CCL2*) or antimicrobial peptides (e.g. *S100A7*) (**Fig 1E**).

I κ B ζ is phosphorylated at multiple sites in the N-terminus

So far, it was unclear if I κ B ζ is exclusively controlled by transcriptional induction of the protein or if its activity is also regulated by posttranslational modifications. As I κ B ζ appeared as two separately migrating bands in Western blot analyses (**Fig 1A** and **1B**), we speculated that I κ B ζ is posttranslationally modified. Using 2D PAGE analysis of endogenous or purified FLAG-tagged I κ B ζ , several I κ B ζ dots, which were separated by their isoelectric properties, could be

detected (**Fig 2A**). The majority of I κ B ζ species shifted to a more acidic pH compared to its calculated isoelectric point of 6.15, suggesting phosphorylation at multiple I κ B ζ sites. Indeed, treatment of lysates with fast alkaline phosphatase led to a disappearance of most I κ B ζ species in the 2D PAGE as well as of the double band in the SDS-PAGE analysis (**Fig 2A and 2B**). To exactly identify the position of the phosphorylation sites in I κ B ζ , we purified FLAG-tagged or endogenous I κ B ζ by immunoprecipitation (IP), digested the protein with trypsin or chymotrypsin, enriched the phosphopeptides with TiO₂ columns and analyzed them by mass spectrometry (MS). In total, we identified nine phosphorylation sites in I κ B ζ , comprising six serine and three threonine residues (**Fig 2C**). The majority of the phosphorylation sites were found in the unstructured N-terminus, which contains in addition to a nuclear localization signal (NLS), a putative transactivation domain (TAD) that has been associated with its transcriptional activity [7, 15]. Two of the identified phosphorylation sites (Ser170 and Ser172) are located within the predicted NLS and thus might affect the nuclear translocation of I κ B ζ . Only one of the phosphorylation sites (Ser578) is located within the C-terminally located ankyrin (ANK) domains, which are important for the binding of NF- κ B members. We noticed that phosphorylation of a cluster of threonine residues (Thr189/Thr193/Thr195) was not only detectable in FLAG-tagged I κ B ζ overexpressing cells, but also in endogenous I κ B ζ upon ZymA stimulation (**Fig 2C and Fig EV2A**). Comparison of I κ B ζ sequences from eight different species, such as *Mus musculus*, *Danio rerio* and *Xenopus tropicalis*, revealed the highest evolutionary conservation in the identified threonine cluster (Thr189/193/195) among the identified phosphorylation sites (**Fig 2D and Fig EV2B**). Interestingly, phosphopeptides comprising this threonine cluster showed mainly phosphorylation at two sites, indicating that I κ B ζ is phosphorylated at multiple sites in the threonine cluster simultaneously (**Fig 2C and Fig EV2C**). To confirm that I κ B ζ is threonine-phosphorylated, we used an anti-phospho-threonine antibody for immunoprecipitation and checked the IP for the presence of I κ B ζ . Indeed, I κ B ζ could be precipitated with the anti-phospho-threonine antibody, which was reversed by phosphatase treatment of the lysate previous to the IP (**Fig 2E**). To investigate the phosphorylation of the threonine cluster in more detail, we generated phospho-specific antibodies against the respective sites. Two of them (Thr189/193) showed phospho-I κ B ζ specific signals, because they recognized wild-type I κ B ζ , but not the phospho-mutant protein (**Fig 2F**). Using these Thr189- and Thr193-specific phospho-I κ B ζ antibodies, we detected, similar to the MS analysis, endogenous I κ B ζ phosphorylation not only in HaCaT cells, but also in A431 cells and in the monocytic cell line THP-1 after ZymA stimulation (**Fig 2G, Fig EV2D and EV2E**).

To understand which kinases are responsible for the Thr189/Thr193/Thr195 phosphorylation, we took advantage of the *in silico* prediction tool iGPS 1.0 and published I κ B ζ phosphorylation predictions [16, 17]. The most likely kinase facilitating I κ B ζ Thr189/Thr193/Thr195

phosphorylation belongs to the mitogen-activated protein kinase (MAPK) family (**Fig 2C**). If MAPKs were responsible for I κ B ζ phosphorylation, we expected them to be activated upon ZymA stimulation. Indeed, increased phosphorylation and hence activation of Erk1/2, JNK1/2 and p38 were observed after 60 and 90 min of ZymA treatment in HaCaT cells (**Fig 2H**). To investigate whether MAPKs play a role in I κ B ζ phosphorylation, we co-expressed I κ B ζ with a hyperactive HRas (G12V) mutant, which triggers the activation of several downstream MAPKs [18]. Both I κ B ζ phosphorylations (Thr189/Thr193), especially the Thr193 phosphorylation, were strongly induced by HRas co-expression (**Fig 2I**). Also, a hyperactive form of MEK1, but not a kinase-inactive mutant, promoted I κ B ζ Thr189 and Thr193 phosphorylation, which correlated with Erk1/2 phosphorylation (**Fig 2J**) [19]. Whereas the B-Raf inhibitor sorafenib led to a strong reduction of I κ B ζ threonine cluster phosphorylation, Erk1/2, JNK1/2 or p38 inhibitors were unable to block Thr193 phosphorylation, suggesting that either another MAPK family member might be responsible for I κ B ζ phosphorylation or that there is redundancy among the MAPK family members (**Fig EV2F** and **EV2G**). Also, I κ B ζ phosphorylation at Ser433, but not at Ser170 or Ser290, was strongly reduced by sorafenib, indicating that not only MAPKs but also other kinases can phosphorylate I κ B ζ (**Fig EV2H**). Interestingly, co-expression of NF- κ B p50, which recruits I κ B ζ to DNA, induced a strong phosphorylation of both Thr189 and Thr193, indicating a potential role of I κ B ζ phosphorylation in the regulation of its transcriptional activity (**Fig EV2I**).

Threonine phosphorylation of I κ B ζ regulates expression of pro-inflammatory genes

To investigate the functional consequences of I κ B ζ phosphorylation at the threonine cluster, we investigated if a non-phosphorylatable mutant was still able to bind known interaction partners. Since the threonine cluster was mainly phosphorylated at two sites simultaneously, we used the I κ B ζ mutant in which all three threonine residues were replaced by alanine for this experiment (Thr189Ala/Thr193Ala/Thr195Ala; 3x T/A). The interaction of I κ B ζ with neither the NF- κ B subunits p50 or p65, the transcription factor STAT3 nor with Akirin2, a protein bridging I κ B ζ to chromatin modifiers, was affected by the mutation of the phosphorylation sites (**Fig 3A-D**) [7, 8, 20, 21]. Next, we tested the transcriptional activity of I κ B ζ in the presence or absence of the threonine phosphorylation sites. On the one hand, we fused the N-terminus of I κ B ζ , which comprises its described TAD [15] and the phosphorylation sites, to a GAL4 DNA-binding domain and measured its capability to drive the expression of a luciferase reporter gene containing five GAL4 binding sites in its promoter. On the other hand, we quantified the induction of a classical NF- κ B reporter by the expression of full-length I κ B ζ wild-type or the non-phosphorylatable mutant alone or in combination with p65 or p50. Neither the GAL4 nor the NF- κ B reporter assay revealed any influence of the threonine phosphorylation in the regulation of the I κ B ζ TAD activity (**Fig 3E** and **Fig EV3A**). To investigate the effect of I κ B ζ phosphorylation on the transcriptome, we used CRISPR/Cas9-generated I κ B ζ knock-out

HaCaT cells, which were reconstituted with doxycycline-inducible I κ B ζ wild-type or the non-phosphorylatable mutant (**Fig 3F**). As measured by an RNAseq, both the I κ B ζ wild-type and the I κ B ζ 3xT/A phospho-mutant expressing HaCaT cells showed a broadly similar induction of target genes in response to ZymA exposure and to doxycycline-driven I κ B ζ expression (**Fig 3G**, **Fig EV3B** and **EV3C**). In accordance with our previous results (**Fig 1E**), induction of I κ B ζ expression alone was sufficient to drive the transcription of selected NF- κ B target genes, such as *S100A7*, *IL23A* or *IL20*, but not of others, such as *TNF*, *IL1B* or *IL17C* (**Fig 3G**). When we filtered the RNAseq data to obtain the most differentially expressed genes between I κ B ζ wild-type or I κ B ζ 3xT/A expressing cells, we identified a first subset of genes that were downregulated in cells containing the non-phosphorylatable I κ B ζ mutant, such as *TLE1*, *GATA6*, *PTP42A*, *CDK6*, *KDM1A*, *HES4*, *YY1* and *ID1*, which are involved in transcriptional regulation, cell cycle control or differentiation. More interestingly, the second set of genes, mostly composed of pro-inflammatory genes such as *IL6*, *CXCL5*, *CCL2*, *CXCL6*, *IL23A* and *S100A9*, exhibited an increased expression if I κ B ζ was phosphorylation-deficient at the threonine cluster (**Fig 3H** and **Fig EV3D**). Since I κ B ζ plays a central role in the induction of pro-inflammatory genes, we focused in the next steps on the validation and characterization of these genes.

HDAC1 recruitment is controlled by I κ B ζ threonine phosphorylation

To confirm that I κ B ζ phosphorylation affects the expression of pro-inflammatory target genes, such as *CXCL5*, *IL23A*, *IL20*, *IL6*, *CCL2* and *S100A9*, we quantified their expression by qPCR and ELISA. Knockout of I κ B ζ strongly inhibited the induction of these target genes after ZymA stimulation (samples without doxycycline), which could be restored by I κ B ζ reconstitution (with doxycycline) (**Fig 1D** and **Fig 4A**). Interestingly, despite very similar expression levels, the non-phosphorylatable mutant of I κ B ζ was more potent in inducing target gene expression compared to the wild-type protein, suggesting an inhibitory function for the I κ B ζ threonine phosphorylation (**Fig 4A**, **Fig 3F** and **Fig EV3D**). In contrast, classical primary NF- κ B target genes such as *NFKBIA* or *NFKBIE* were induced by ZymA treatment but were independent of I κ B ζ phosphorylation (**Fig 4A** and **Fig EV4A**). The increased ability of the I κ B ζ 3xT/A mutant to induce a subset of pro-inflammatory target genes was also confirmed on protein level, because we detected elevated CXCL5 and IL-6 levels in the supernatants of ZymA-stimulated cells compared to I κ B ζ wild-type expressing cells (**Fig 4B** and **Fig EV4B**). Thus, these data suggest an anti-inflammatory role of I κ B ζ threonine phosphorylation. At first glance, it seems counterintuitive that MAPKs should be responsible for I κ B ζ phosphorylation, because they are mainly considered as pro-inflammatory kinases. Under certain conditions, however, anti-inflammatory effects by several MAPK members, such as p38 or ERK have been reported [22-24].

Since I κ B ζ 3xT/A expressing cells showed increased target gene expression, we speculated that the termination of transcription might be induced by I κ B ζ phosphorylation. We hypothesized that HDAC1 might be recruited by phosphorylated I κ B ζ and thus limit target gene expression, as it has been described for the NF- κ B member p65 [6, 25]. Indeed, pharmacological inhibition of HDAC1 resulted in an enhanced expression of *IL6*, *IL20* and *S100A9* in ZymA-stimulated HaCaT cells (**Fig 4C**). Next, we tested the capacity of I κ B ζ to recruit HDAC1 in co-immunoprecipitation experiments. Whereas wild-type I κ B ζ interacted with HDAC1 in ZymA-stimulated HaCaT cells, the I κ B ζ 3xT/A mutant exhibited a strong defect in HDAC1 binding (**Fig 4D**). Accordingly, we also detected HDAC1 recruitment by I κ B ζ to the promoter of target genes, such as *IL6* and *IL23A* which was decreased in cells expressing the non-phosphorylatable I κ B ζ mutant (**Fig 4E**). The concept that I κ B ζ plays a crucial role not only in the induction, but also in the termination of target gene transcription has been discussed previously. In macrophages and dendritic cells, I κ B ζ is not only required for the induction of IL-6 after LPS treatment, but also for the resolution of inflammation [26]. In this context, I κ B ζ was found to recruit Tet2, a dioxygenase involved in DNA methylation, to the *IL6* promoter, which in turn represses *IL6* transcription via the recruitment of HDAC1/2 [26]. Here, we report that I κ B ζ can interact with HDAC1 and recruit it to the promoters of I κ B ζ target genes in keratinocytes. Moreover, the inhibition of HDAC1/2 led to an increased expression of I κ B ζ target genes, such as *IL6*, *IL20* and *S100A9*, confirming the role of these HDACs in terminating the transcription of these genes. In contrast to myeloid cells, we were unable to detect an I κ B ζ -Tet2 interaction in keratinocytes, indicating that the resolution of I κ B ζ -mediated inflammation might be differentially regulated in this cell type. We propose I κ B ζ phosphorylation as a mechanism to terminate the transcription of a subset of NF- κ B target genes. Since I κ B ζ is essential for the development of psoriasis and for the survival of Diffuse Large B-Cell Lymphoma, it will be interesting to monitor I κ B ζ posttranslational modifications in these diseases [9, 27].

In conclusion, we provide several pieces of evidences for an important role of I κ B ζ and its post-translational modification status in the immune response of keratinocytes during fungal recognition. (I) ZymA treatment of keratinocytes leads to a strong induction of I κ B ζ at the protein level. (II) Silencing or overexpression of I κ B ζ modulates the ZymA-induced expression of important immune modulators, such as *IL6*, *IL1B* or *S100A9*. (III) I κ B ζ threonine cluster phosphorylation can be detected by MS analyses and phospho-specific I κ B ζ antibodies in ZymA-stimulated keratinocytes. (IV) Activation of MAPK correlates with the strength of I κ B ζ phosphorylation. (V) Inhibition of I κ B ζ threonine cluster phosphorylation reduces HDAC1 recruitment and thus the optimal termination of target gene transcription. Collectively, this study highlights a novel layer of complexity in the regulation of pro-inflammatory genes by the atypical I κ B member I κ B ζ .

Material and Methods

Isolation, cultivation and stimulation of human primary keratinocytes

Human primary keratinocytes were isolated from foreskin of children immediately after circumcision. To obtain single cells, the skin tissue was incubated with 10 mg/ml dispase (Roche) overnight followed by trypsination and filtration. Primary keratinocytes from at least 3 donors were pooled and cultivated in CnT-BM.1 medium supplemented with the CnT-07 Supplement Pack and gentamycin (all from CELLnTEC). For stimulation, primary keratinocytes were used at passage 2 and at confluence of 70-90%. HaCaT and A431 cells were purchased from ATCC and maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum and antibiotics (penicillin/streptomycin). For stimulation, cells were treated with 200 µg/ml Zymosan A (Sigma-Aldrich). Cells were harvested by trypsination, washed with PBS and lysed in Tris-NaCl lysis buffer containing 1% Triton X-100, protease inhibitors (Complete; Roche) and phosphatase inhibitors (50 mM NaF, 10 mM Na₄P₂O₇ and 10 mM Na₃VO₄).

SDS-PAGE analysis and two-dimensional electrophoresis

Western blot analysis was performed as previously described [2]. The following primary antibodies were used for protein detection: anti-IκBζ (Cell Signaling, #9244), anti-JNK1/2 (Cell Signaling, #9285), anti-p-JNK1/2 (Cell Signaling, #4668), anti-p-ERK1/2 (SCBT, sc-7383), anti-ERK1/2 (SCBT, sc-514302), anti-p-p38 (Cell Signaling, #4511), anti-p38 (Cell Signaling, #8690), anti-HDAC1 (Cell Signaling, #5356) and anti-GAPDH (Cell Signaling, #2118). As secondary antibodies HRP-coupled anti-mouse or anti-rabbit antibodies (Jackson ImmunoResearch) were used. For protein dephosphorylation, cells were lysed in standard lysis buffer lacking phosphatase inhibitors and treated with a thermosensitive alkaline phosphatase (FastAP, Thermofisher) for 1 hour at 37°C. Two-dimensional electrophoresis was performed utilizing the Bio-Rad ReadyPrep 2-D starter kit according to the manufacturers' protocol using an IP-Strip ranging from pH 4-7. Electrophoresis in the second dimension was carried out using 13 % SDS-PAGE gels.

Chromatin immunoprecipitation

ChIP was basically done as described before [10]. To increase protein-protein cross-linking, cells were treated with DGS together with 1% PFA. The following antibodies were used: anti-HDAC1 (Diagenode, C15410325), anti-FLAG (Sigma, F1804) or IgG control antibody (Abcam, ab171870). The following primers were used for detection of HDAC1 binding:

IL6 AGGGAGAGGGAGCGATAAAC
IL23 TCTAGCCACAGCAACCACAC

GTCAAAGGAGGACCTTGTGG
ACTCACAGCAGGTGGGATTC

Plasmids and transient transfection

T189A/T193A/T195A mutant was generated by standard double PCR using FLAG-tagged wild-type I κ B ζ as a template and cloned in to a pCR3 standard vector. STAT3 (Addgene, #8706), STAT3C (Addgene, #8722), p50 and p65 (kindly provided by Dr. Thome, University of Lausanne) were subcloned into a Strep-tagged pCR3 construct. The Gal4/3 luciferase reporter construct was kindly provided by Dr. Schmitz, University of Marburg). Gal4-I κ B ζ constructs were generated by PCR amplification of the region corresponding to the first 442 amino acids of FLAG-I κ B ζ WT or FLAG-I κ B ζ 3xT/A mutant.

CRISPR/Cas9 gene editing and generation of I κ B ζ Tet-ON system

The I κ B ζ KO HaCaT cells were generated using the CRISPR/Cas9 one vector system according to the published protocol [28]. The guide RNA with the sequence TCGGGTAAAGAACTCAGTGA against I κ B ζ was cloned into the lentiCRISPRv2 containing Cas9 (Addgene #52961). After lentiviral transduction, cells were selected using 1 μ g/mL puromycin and single clones were screened for lack of I κ B ζ expression. FLAG-I κ B ζ WT and FLAG-I κ B ζ 3xT/A mutant were cloned into a lentiviral pInducer20 plasmid (Addgene #44012) using the pENTR TOPO cloning system (Invitrogen). After lentiviral particles production in HEK293T cells, the I κ B ζ KO HaCaT cells were transduced and selected with 450 μ g/mL G418 (Invivogen). Induction of I κ B ζ expression cells was achieved with doxycycline treatment (2 μ g/ml, AppliChem) for 24 hours.

Luciferase and Gal4 reporter assay

The respective reporter constructs were transiently transfected into HEK293T cells. 24 hours after transfection cells were lysed in passive lysis buffer and the dual luciferase assay was performed according to the manufacturers' protocol (Promega).

Immunoprecipitation assays

Cells were lysed in standard lysis buffer and sonicated for 5 minutes. Protein concentrations of cell extracts were adjusted with lysis buffer and lysates were precleared with sepharose 6B (Sigma-Aldrich) for one hour at 4°C. Precleared lysates were incubated either with anti-FLAG M2 agarose beads (Sigma), with Streptavidin beads (IBA), or with antibodies specific for HDAC1 (Cell Signaling), p-threonine (Cell Signaling) or I κ B ζ (Cell Signaling) for one hour at 4°C. For endogenous IPs immune complexes were precipitated with protein G sepharose (GE Healthcare) and eluted by 3x SDS-PAGE sample buffer.

RNA isolation and qPCR

RNA was isolated using the RNeasy Mini Kit, according to the manufacturer's protocol (Qiagen). Reverse transcription was performed the Biozym cDNA Synthesis Kit with hexamers primers (Biozym). cDNA levels were measured by real-time PCR with the GreenMasterMix (Genaxxon). qPCR was carried out in 384-well plates on a LightCycler 480 II system (Roche). Gene expression was quantified with the following primers:

<i>RPL37A</i>	AGATGAAGAGACGAGCTGTGG	CTTTACCGTGACAGCGGAAG
<i>IL6</i>	CATCCTCGACGGCATCTCAG	TGCCTCTTTGCTGCTTTCAC
<i>CCL2</i>	ATAGCAGCCACCTTCATTCCC	AGATCTCCTTGGCCACAATGG
<i>CXCL5</i>	AGCTGCGTTGCGTTTGTTTAC	TGGCGAACACTTGCAGATTAC
<i>CXCL6</i>	AGAGCTGCGTTGCACTTGTT	GCAGTTTACCAATCGTTTTGGGG
<i>IL23A</i>	CTCAGGGACAACAGTCAGTTC	ACAGGGCTATCAGGGAGCA
<i>NFKBIA</i>	CTCCGAGACTTTCGAGGAAATAC	GCCATTGTAGTTGGTAGCCTTCA
<i>NFKBIE</i>	TCTGGCATTGAGTCTCTGCG	AGGAGCCATAGGTGGAATCAG
<i>S100A9</i>	GGTCATAGAACACATCATGGAGG	GGCCTGGCTTATGGTGGTG
<i>IL20</i>	ATGAAAGCCTCTAGTCTTGCCCT	GCCCCGTATCTCAGAAAATCC
<i>IL1B</i>	ATGATGGCTTATTACAGTGGCAA	GTCGGAGATTTCGTAGCTGGA
<i>S100A7</i>	ACGTGATGACAAGATTGACAAGC	GCGAGGTAATTTGTGCCCTTT
<i>JunD</i>	TCATCATCCAGTCCAACGGG	TTCTGCTTGTGTAAATCCTCCAG
<i>CFS2</i>	TCCTGAACCTGAGTAGAGACAC	TGCTGCTTGTAGTGGCTGG

Relative mRNA levels were normalized to the reference gene RPL37A using the $2^{-\Delta\Delta CT}$ method [29].

ELISA

Human CXCL5 and IL-6 levels were quantified using the human Ready-SET-Go ELISA kit (eBioscience) according to the manufacturer's protocol.

Mass Spectrometry

Gel bands of purified I κ B ζ were subjected to in-gel digestion as previously described [30]. After digestion, peptides were separated by nano-flow reversed-phase liquid chromatography coupled to Q Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific). In brief, samples were reconstituted in 5% DMSO (v/v) and 5% (v/v) Formic Acid (FA) and peptides were loaded on a C18 PepMap100 pre-column (300 μ m i.d. x 5 mm, 100Å, Thermo Fisher Scientific) at a flow rate of 12 μ L/min in 100% buffer A (0.1% FA in water). Peptides were then transferred to an in-house packed analytical column heated at 45°C (75 μ m i.d. x 50 cm, packed with ReproSil-Pur 120 C18-AQ, 1.9 μ m, 120 Å, Dr. Maisch GmbH) and separated using a 60 min gradient from 10 to 30% buffer B (0.1% FA in acetonitrile) at a flow rate of 200 nL/min. Q Exactive survey scans were acquired at 70,000 resolution to a scan range from 350 to 1500 m/z, AGC target 3e6, maximum injection time 50 ms. The mass

spectrometer was operated in a data-dependent mode to automatically switch between MS and MS/MS. The 10 most intense precursor ions were submitted to HCD fragmentation using an MS/MS resolution set to 17 500, a precursor AGC target set to 5e4, a precursor isolation width set to 1.5 Da, and a maximum injection time set to 120 ms. Raw data files were processed for protein identification using MaxQuant, version 1.5.0.35, integrated with the Andromeda search engine as described previously [31-33]. The MS/MS spectra were searched against the human Uniprot database; precursor mass tolerance was set to 20 ppm and MS/MS tolerance to 0.05 Da. Enzyme specificity was set to either trypsin with a maximum of two missed cleavages or chymotrypsin with a maximum of nine missed cleavages. False discovery rate for protein and peptide spectral matches was set at 0.01.

RNAseq

Libraries were constructed with the Ultra RNA Library Prep Kit at the Core Facility Genomics in Münster, Germany. Sequencing was performed using the IlluminaNextSeq High Output kit. The HISAT2 program was used to map sequences against the human reference genome hg19. From raw gene counts, differentially expressed genes were computed using the Bioconductor DESeq2. Significantly deregulated genes of I κ B ζ WT and I κ B ζ 3xT/A HaCaT cells presented a corrected p-value ≤ 0.05 and an absolute fold change (FC) > 1 . To identify all differentially expressed genes (DEG) of I κ B ζ 3xT/A compared to I κ B ζ WT cells, genes that followed p-value < 0.05 were filtered, independently of absolute FC.

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Author contributions

PG, CS, PB, AS, BS, SL and AM performed experiments; PG, SM, AH, BS, DK, KS and SH analyzed data; PG, KS and SH wrote the manuscript which all other authors commented on; SH conceived and coordinated the study.

Competing financial interests

None

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Figure legends

Figure 1. Zymosan A induces I κ B ζ and I κ B ζ -dependent target gene expression in keratinocytes.

A) Immunoblot analysis of human primary KCs treated for 1.5 hours with *S. aureus* and *E. coli* (2×10^8 CFU/ml living bacteria), PMA/Iono (80 ng/ml PMA, 1 μ M ionomycin), PMA (800 ng/ml), peptidoglycan (10 μ g/ml), IL-17A (100 ng/ml), TNF α (1 ng/ml), IL-17A + TNF α , IL-1 β (10 ng/ml) or ZymA (200 μ g/ml). **B)** I κ B ζ protein levels in human primary keratinocytes and HaCaT cells treated for the indicated time points with ZymA. **C)** qPCR analysis of human primary KC stimulated with ZymA for 2, 4, 6 and 8 hours. **D)** Primary KCs were lentivirally transduced with a control or I κ B ζ -specific shRNA and stimulated with ZymA for 2 hours before target gene expression was measured by qPCR. I κ B ζ silencing was confirmed by Western blotting. **E)** CRISPR/Cas9 I κ B ζ KO HaCaT cells were reconstituted with an empty vector control (Empty Tet-ON) or FLAG-tagged I κ B ζ (I κ B ζ Tet-ON). I κ B ζ expression was induced by adding doxycycline for 24 hours and controlled by Western blotting. Target gene expression was assessed by qPCR. **C-E)** Transcript levels were normalized to the untreated sample. Error bars correspond to the mean \pm SD. Statistical significance was calculated using t-test (* $p < 0.05$, ** $p < 0.01$). **A-E)** Data is representative of at least three independent experiments.

Figure 2. I κ B ζ is phosphorylated at multiple residues.

A) 2D-PAGE analysis of A431 cells stimulated with ZymA for 1 hour or HEK293T cells expressing FLAG-tagged I κ B ζ . Lysates were either left untreated (Co), or treated for 1 hour with alkaline phosphatase (FAP). **B)** Immunoblot analysis of HaCaT cells that were left untreated or stimulated with ZymA for 1 hour. After cell lysis in the presence or absence of phosphatase inhibitors, cell lysates were incubated with or without FAP. p-Erk1/2 protein was used as a positive control for successful FAP treatment. **C)** Schematic representation of the 9 phosphosites identified by LC-MS/MS and the respective kinases predicted by *in silico* analysis. The nuclear localization sequence (NLS), the transactivation domain (TAD) and the ankyrin repeats (ANK) of I κ B ζ are indicated. Table depicting peptide sequences from mass-spectrometry analysis, the localization probability and the MaxQuant identification score. ‡, chymotrypsin digestion. **D)** Evolutionary conservation of identified phosphosites among I κ B ζ protein sequences from *Danio rerio*, *Xenopus tropicalis*, *Gallus gallus*, *Mus musculus*, *Bos taurus*, *Felis catus*, *Equus caballus* and *Homo sapiens*. **E)** Proteins containing p-threonine residues were immunoprecipitated from cell lysates of untreated (Co) or FAP treated samples and I κ B ζ presence was analysed Western blot analysis. **F)** Validation of phospho-specific antibodies generated against I κ B ζ p-Thr189 and p-Thr193 by Western blotting. The I κ B ζ 3xT/A phospho-mutant (Thr189Ala/Thr193Ala/Thr195Ala) was used as a specificity control. **G)**

Immunoprecipitated I κ B ζ from HaCaT cells were analysed for Thr189 and Thr193 phosphorylation. The heavy chain (h.c.) of the antibody was used as IP control. **H)** Immunoblot analysis of MAP kinase activation in HaCaT cells stimulated with ZymA for the indicated time points. **I)** HEK293T cells were transfected to express FLAG-tagged I κ B ζ and FLAG-HRas G12V and analysed by immunoblotting with the indicated antibodies. **J)** FLAG-tagged I κ B ζ was expressed in combination with HA-tagged wild-type, constitutively active (R4F) or catalytically inactive MEK1 (K97M). The phosphorylation status of Erk 1/2 was analysed as control. Data is representative of at least three (A, B, G-J) or two (E, F) independent experiments.

Figure 3. Phosphorylation of I κ B ζ at Thr189/Thr193/Thr195 negatively regulates a set of target genes.

A-C) Phosphorylation of I κ B ζ at the threonine cluster does not alter binding to known interaction partners. Co-immunoprecipitation of FLAG-tagged I κ B ζ WT or I κ B ζ 3xT/A with Strep-tagged versions of p50 (**A**), p65 (**B**), wild-type STAT3 or hyperactive STAT3C mutant (**C**) was analysed by Western blotting. The heavy chain (h.c.) of the IP antibody is indicated **D)** Strep-tagged I κ B ζ WT and I κ B ζ 3xT/A were expressed with or without FLAG-tagged Akirin2, which was immunoprecipitated using anti-FLAG beads. **E)** NF- κ B luciferase reporter assay of HEK293T cells transfected with FLAG-tagged I κ B ζ WT or I κ B ζ 3xT/A together with p50, p65 or an empty vector control. Mean values \pm SD of NF- κ B activity were normalized to the vector control and are given as relative light units (RLU). **F-H)** I κ B ζ KO HaCaT cells were reconstituted with an inducible expression plasmid coding for either FLAG-I κ B ζ WT or FLAG-I κ B ζ 3xT/A and were treated with solvent or doxycycline for 22 hours and then incubated for further 2 hours with or without ZymA as indicated. **F)** Immunoblot control of FLAG-I κ B ζ WT and FLAG-I κ B ζ 3xT/A protein expression. **G)** Heatmap of gene sets differentially expressed in solvent or ZymA treated HaCaT cells; untreated (Solvent, -), doxycycline treated (Solvent, +), and both doxycycline and ZymA (Solvent, +). Only genes with a fold change (FC) > 2 and a p-value < 0.05 are shown. **H)** Heatmap of differentially expressed genes between doxycycline-induced I κ B ζ WT and I κ B ζ 3xT/A HaCaT cells. Gene sets were selected with a p-value < 0.05. **A-E)** Data is representative of three independent experiments. **G-H)** Total RNA was isolated and samples were analysed by RNAseq in biological triplicates.

Figure 4. Mutation of the I κ B ζ threonine phospho-cluster results in reduced recruitment of HDAC1.

A) qPCR quantification of mRNA expression of I κ B ζ target genes. HaCaT cells were cultivated for 22 hours in the absence or in the presence of doxycycline to induce the expression of I κ B ζ WT or I κ B ζ 3xT/A and then incubated for further 2 hours with or without ZymA. Results show

mean values of mRNA expression from biological triplicates, which were normalized to the mRNA levels from untreated IκBζ WT cells. **B)** HaCaT cells inducibly expressing IκBζ WT or IκBζ 3xT/A were treated with or without doxycycline for 24 hours and then stimulated for 16 hours with solvent or ZymA. Culture supernatants were analysed by ELISA for CXCL5 and IL-6 concentration. Secretion was normalized to untreated IκBζ WT cells. **C)** qPCR analysis of HaCaT cells carrying Tet-inducible IκBζ WT untreated or treated with Dox for 22 hours and ZymA-stimulated for 2 hours with or without the HDAC1/3 inhibitor MS-275 (10 μM). **D-E)** Wild-type FLAG-IκBζ or FLAG-IκBζ 3xT/A was induced by doxycycline treatment for 22 hours and HaCaT cells were stimulated 2 hours with ZymA. **D)** Cell extracts were subjected to immunoprecipitation with HDAC1 antibody. The heavy chain (h.c.) of the IP antibody is indicated. **E)** ChIP analysis from FLAG-IκBζ WT or FLAG-IκBζ 3xT/A expressing HaCaT cells. HDAC1 binding to *IL6* and *IL23A* promoters was compared to the IgG control. (A, B, C, E) Bars correspond to the mean ± SD. Statistical significance was calculated using t-test (*p < 0.05, **p < 0.01). Data is representative of at least three (A, B, D, E) or two (C) independent experiments.

Figure EV1. IκBζ expression in SCC cell lines and CRISPR/Cas9-generated IκBζ knock-out HaCaT single cell clones upon ZymA stimulation.

A) Immunoblot analysis of human squamous cell carcinomas and head and neck squamous cell lines treated with ZymA (200 μg/ml) for the indicated time. **B)** HaCaT cells were transduced with a CRISPR vector containing a guide RNA specific for the first exon of *NFKBIZ*. Several CRISPR KO single cell clones were isolated by limiting dilution and stimulated with ZymA to verify the IκBζ knockout by Western blotting. Clone G4 was used for subsequent experiments.

Figure EV2. IκBζ phosphorylation at the threonine phospho-cluster.

A) Immunoblot analysis of immunoprecipitated endogenous IκBζ from A431 cells stimulated for 1.5 hours with ZymA (200 μg/ml). **B)** Alignment of IκBζ protein sequences within the region of the threonine phosphorylation cluster from the indicated species. **C)** MS/MS spectra showing phosphorylation of endogenous IκBζ Thr189 and Thr193/195. **D-E)** Immunoblot analysis of A431 (**D**) and THP-1 cells (**E**), either left untreated or stimulated with ZymA for 1 hour. (**A, D-E**) IκBζ antibody was used for immunoprecipitation (IP) of endogenous IκBζ. **F-H)** HEK293T cells expressing Strep-tagged IκBζ, FLAG-tagged p50 and FLAG-tagged H-Ras G12V. 2 days after transfection cells were treated with sorafenib (10 μM) for 1 hour followed by Strep-pulldown. IκBζ phosphorylation was visualized by Western blotting (**F**) or MS analysis (**G-H**). **I)** Immunoblot analysis of HEK293T cells transfected with FLAG-tagged IκBζ with or without FLAG-tagged p50.

Figure EV3. Effect of the 3xT/A mutant on I κ B ζ transactivation activity and target gene expression.

A) Gal4 luciferase reporter assay of HEK293T transfected with an empty vector or increasing amounts of Gal4 constructs fused to p65, or the N-terminus of I κ B ζ WT or I κ B ζ 3xT/A. Gal4 luciferase promoter activity is shown in relative light units (RLU) normalized to an internal Renilla luciferase control. Protein expression levels were tested by Western blotting. **B)** Principle component analysis of the RNAseq analysis. The symbols correspond to the different condition as indicated. **C)** Heatmap showing gene sets upregulated upon ZymA stimulation in untreated (without doxycycline) I κ B ζ WT and I κ B ζ 3xT/A HaCaT cells. Selected genes show a fold change (FC) > 2 and a p-value < 0.05. **D)** Heatmap of genes differentially expressed between doxycycline induced I κ B ζ WT and I κ B ζ 3xT/A HaCaT cells stimulated with ZymA. Gene sets were selected with a p-value < 0.05.

Figure EV4. Effect of the 3xT/A mutant on I κ B ζ target gene expression.

A) qPCR analysis of *NFKBIE* expression in I κ B ζ WT or I κ B ζ 3xT/A expressing HaCaT cells treated with ZymA as indicated. **B)** Immunoblot analysis of HaCaT cells used for RNAseq analysis. Cell expressing doxycycline-inducible I κ B ζ WT or I κ B ζ 3xT/A were either left untreated or Dox-treated for 24 hours and then further incubated 16 hours in the absence or presence of ZymA.