Role of Monoubiquitylation on the Control of IκBα Degradation and NF-κB Activity

Elisa Da Silva-Ferrada1, Mónica Torres-Ramos1,², Fabienne Aillet1, Michela Campagna2, Carlos Matute3, Carmen Rivas2, Manuel S. Rodríguez1,⁴, Valérie Lang1

1Proteomics Unit, CIC bioGUNE, CIBERehd, Bizkaia Technology Park, Derio, Spain, 2Centro Nacional de Biotecnología, CSIC, Campus Universidad Autónoma de Madrid, Madrid, Spain, 3Department of Neuroscience University of the Basque Country UPV/EHU, CIBERNED, Leioa, Bizkaia, Spain, 4Department of Biochemistry University of the Basque Country UPV/EHU, Leioa, Bizkaia, Spain

Abstract

The NF-κB pathway is regulated by multiple post-translational modifications including phosphorylation, ubiquitylation and SUMOylation. Many of these modifications act on the natural inhibitor IκBα modulating its capacity to control signal-mediated NF-κB activity. While the canonical pathway involving the phosphorylation and polyubiquitylation of IκBα has been well characterized, the role of these post-translational modifications in the control of basal NF-κB activity has not been deeply explored. Using the recently developed Tandem-repeated Ubiquitin Binding Entities (also known as ubiquitin traps) to capture ubiquitylated proteins, we identified monoubiquitylated forms of IκBα from multiple rat organs and cell types. The identification of these forms was demonstrated through different procedures such as immunoprecipitations with specific ubiquitin antibodies or His6-Ubiquitin pull downs. Monoubiquitylated forms of IκBα are resistant to TNFα-mediated degradation and can be captured using TUBE5, even after proteasome inhibitors treatment. As it occurs for monoSUMOylation, monoubiquitylation is not dependent of the phosphorylation of IκBα on the serines 32/36 and is not optimally degraded after TNFα stimulation. A ubiquitin-IκBα fusion exhibits phosphorylation defects and resistance to TNFα mediated degradation similar to the ones observed for endogenous monoubiquitylated IκBα. The N-terminal attachment of a single ubiquitin moiety on the IκBα fusion results in a deficient binding to the IκBα/B kinase and recruitment of the SCF ligase component ITBCP, promoting a negative impact on the NF-κB activity. Altogether, our results suggest the existence of a reservoir of monoubiquitylated IκBα resistant to TNFα-induced proteolysis, which is able to interact and repress DNA binding and NF-κB transcriptional activity. Such pool of IκBα may play an important role in the control of basal and signal-mediated NF-κB activity.

Introduction

The nuclear factor κB (NF-κB) is a family of transcription factors that regulate the expression of various genes involved in inflammatory, anti-apoptotic and immune responses [1][2]. The NF-κB pathway can be activated by many different extracellular signals that induce multiple post-translational modifications such as phosphorylation, ubiquitylation and SUMOylation, acting at various levels of the signaling cascade [3–5]. As many other stimuli, the pro-inflammatory cytokine TNFα (tumor necrosis factor-alpha) ends with the activation of the IKK (IκBα Kinase) complex, composed by IKKα, IKKβ and IKKγ/NEMO [6][7]. IKK phosphorylates the alpha inhibitor of NF-κB, IκBα, on the serines 32 and 36 and targets it for ubiquitylation at the main ubiquitylation sites, lysine 21 and 22 by a SCF (Skp, Cullin, F-box) ubiquitin ligase complex containing the beta-transducin repeat-containing protein ITBCP [8][9]. The presence of the DSGXXS motif determines the specific interaction of ITBCP with the phosphorylated Inhibitor of NF-κB alpha (IκBα), which is crucial for its ubiquitylation and posterior proteasome degradation. In contrast, the conjugation with the small ubiquitin-like modifier 1 (SUMO-1) is not dependent on the phosphorylation on the serines 32 and 36 of IκBα and has a positive impact on IκBα stability [10]. Ubiquitylation of IκBα is tightly controlled by the action of unidentified DUBs (de-ubiquitylating enzymes). Released NF-κB is then imported to the nucleus where it activates the transcription of a large number of genes including IκBα and TNF-receptor 2 [11][2]. Newly synthesized IκBα is imported into the nucleus where it ends up with NF-κB mediated transcription by detaching it from DNA promoter sequences and favoring its export to the cytoplasm [12][13]. In this study, the use of ubiquitin traps (TUBE5s for Tandem-repeated Ubiquitin Binding Entities) [14] allowed us to identify monoubiquitylated IκBα from rat organs, as well as from different...
cell lines. Using in vitro and ex vivo approaches we aimed to understand the impact that a single ubiquitin moiety can have on the properties and inhibitory capacity of IkBx. The evidence presented here suggests the existence of a pool of monoubiquitylated IkBx resistant to degradation whose function might play an important role in the control of basal and signal-induced NF-κB activity.

Results

Presence of monoubiquitylated IkBx in organs and cell lines

The recently developed ubiquitin-traps (TUBEs) that specifically capture ubiquitin and ubiquitylated proteins [14] were adapted to extract ubiquitylated proteins from rat organs. As reported, TUBEs capture preferentially polyubiquitylated proteins, however monoubiquitylated proteins can also be captured when abundantly expressed [14]. Monoubiquitylated IkBx can be easily detected by Western blot in a mix of total ubiquitylated proteins purified by TUBEs from liver, heart, brain, muscle, lung and kidney (Figure 1), suggesting a function for this form of IkBx in normal tissues. Monoubiquitylated IkBx can also be captured using a similar procedure with multiple cell lines such as HEK293 (Figure 2A), Jurkat (Figure 2B) and HeLa (data not shown). The identification of the monoubiquitylated IkBx was confirmed using several protocols including immunoprecipitations with a specific anti-ubiquitin antibody of the TUBE-captured material. Under these conditions endogenous and exogenous monoubiquitylated IkBx can be detected using anti-IkBx, anti HA or anti SV5 antibodies, respectively (Figure 2C). Furthermore, monoubiquitylated IkBx can be also detected in cells co-transfected with plasmids encoding histidylated versions of ubiquitin with or without vectors expressing IkBx WT to purify exogenous and endogenous ubiquitylated IkBx respectively (Figure 2D). Monoubiquitylated IkBx can also be easily reproduced in vitro using an ubiquitin mutant (Ub KO) where all reactive lysine residues have been changed to arginine (Figure 2E). However, monoubiquitylated IkBx cannot be immunoprecipitated with monoclonal or polyclonal IkBx antibodies, alone or combined in a TUBE-IkBx immunoprecipitation procedure (Figure S1 and S2). Under these conditions monoubiquitylated IkBx is detected in the unbound fraction. Thus, the monoubiquitylated form of IkBx, found in organs and cell lines, shows a poor accessibility to IkBx immunoprecipitation but can be detected using denaturing gels followed by Western blot analysis.

Monoubiquitylated IkBx is not sensitive to the TNFα-mediated degradation

To evaluate the susceptibility of the monoubiquitylated form of IkBx to be degraded by TNFα a TUBE-capture assay was performed in HEK293 cells treated or not with proteasome inhibitor MG132 (Figure 3). As expected, most IkBx is degraded after 20 minutes of TNFα-stimulation as it can be seen in the input (IN). This proteolytical process is blocked in the presence of MG132 where IkBx is accumulated as hyperphosphorylated form. The analysis of the TUBE-captured material shows that monoubiquitylated IkBx remain very stable after 20 or 60 minutes of TNFα stimulation even in the presence of proteasome inhibitor (Figure 3A). Interestingly, the capacity of the TUBE-hHR23 to capture monoubiquitylated and polyubiquitylated forms of IkBx is not compromised when the proteasome activity is inhibited. These results suggest that the monoubiquitylated form of IkBx is not destabilized by the induction with TNFα but it is slightly accumulated after treatment with MG132 (Figure 3A). To evaluate the role of the serines 32/36 phosphorylation on the accumulation of monoubiquitylated form of IkBx, a mutant S32/36A was transfected into HEK293 cells. In the absence of TNFα stimulation and MG132, modified forms of IkBx were captured
using His6-Ubiquitin, His6-SUMO-1 or His6-SUMO-2 and nickel beads chromatography. Our results confirm that the monoubiquitylation of IκBα is not dependent of the phosphorylation of serines 32 and 36 (Figure 3B). MonoSUMOylation with SUMO-2 and SUMO-1 (only visible on long exposures, data not shown) are also independent of this signaling pathway. In contrast, high molecular weight forms can not be seen on the S32/36A IκBα mutant after TNFα stimulation in a situation where polyubiquitylated IκBα WT is well accumulated (Figure 3B).

Extended half-life of monoubiquitylated IκBα

The analysis of a subpopulation of IκBα molecules and in particular its impact on NF-κB activity is difficult to achieve if mixed populations of IκBα molecules are present. There is no available method of purification able to isolate unmodified or ubiquitylated IκBα molecules with homogeneous characteristics. For this reason, to further understand the role of monoubiquitylated form of IκBα, an ubiquitin-IκBα fusion protein was generated (Figure 4A). This approach has been largely used as it can be judged in the literature especially to study the role of ubiquitin and ubiquitin-like proteins in the regulation of protein localization and function [15]. The ubiquitin-IκBα fusion has been optimized to resist to the action of DUBs by introducing, at the C-terminal of ubiquitin, a double alanine (AA) instead of the double glycine (GG). To avoid additional attachment of moieties at the N-terminus of IκBα, lysine 21 and 22 were mutated to alanine (KK to AA). Attachment of ubiquitin at a single N-terminus lysine acceptor of IκBα provide similar stability effects [16,17]. Ubiquitin-IκBα fusion protein shows similar sub-cellular distribution than IκBα WT (Figure S3). When expressed in HEK293 and HeLa cells, ubiquitin-IκBα fusion protein showed an extended half-life compared to IκBα WT (Figure 4B and data not shown). The effect of a single ubiquitin moiety on IκBα stability is also reflected after signal-mediated stimulation, as this ubiquitin-IκBα fusion shows resistance to TNFα induced degradation (Figure 4C). A kinetic of degradation was performed to confirm that the observed resistance was not due to a delay in TNFα-induced IκBα degradation (Figure 4D). Proteolytical defects are not due to the...
mutation of the Lysine 21 or/and 22 since its presence in other ubiquitin-IκBα fusions provides similar results (data not shown). Furthermore, these effects appear to be specific of ubiquitin, as fusions containing other molecules from the ubiquitin family do not provide the same results (data not shown). Thus, from these results we conclude that the attachment of a single ubiquitin moiety extends the half-life of the ubiquitin-IκBα fusion and perfectly reproduce the stability after TNFα-stimulation observed with the endogenous monoubiquitylated form of IκBα.

**Phosphorylation defects of monoubiquitylated IκBα**

In order to understand the molecular origin of ubiquitin-IκBα fusion stability, its capacity to be phosphorylated after TNFα stimulation was investigated. We could observe a reproducible reduction of ubiquitin-IκBα fusion phosphorylation when compared to IκBα WT (Figure 5A). This is mainly due to the incapacity of ubiquitin-IκBα fusion protein to efficiently bind IKKβ compared to IκBα WT in TNFα-stimulated HEK293 cells (Figure 5B). Experimental data demonstrate that the exogenously expressed...
ubiquitin-\(\text{I}k\)\(\text{B}\)\(\alpha\) fusion also fails to efficiently interact with \(\beta\)TrCP compared to \(\text{I}k\)\(\text{B}\)\(\alpha\) WT under the same experimental conditions. Altogether our results clearly indicate that ubiquitin-\(\text{I}k\)\(\text{B}\)\(\alpha\) fusion but not \(\text{I}k\)\(\text{B}\)\(\alpha\) WT shows defects in the interaction with critical molecules of the signaling pathway including IKK\(\beta\) and \(\beta\)TrCP, thus explaining at least in part, its resistance to proteolysis (Figure 5B).

**Figure 4. Stability of ubiquitin-\(\text{I}k\)\(\text{B}\)\(\alpha\) fusion protein.** A) Ubiquitin-\(\text{I}k\)\(\text{B}\)\(\alpha\) fusion was generated with an N-terminal HA and C-terminal V5 epitopes. B) HEK293 cells transfected with \(\text{I}k\)\(\text{B}\)\(\alpha\) WT and ubiquitin-\(\text{I}k\)\(\text{B}\)\(\alpha\) fusion were treated with 50 \(\mu\)g/ml CHX for the indicated times. The graph corresponds to the mean of three independent experiments. C) \(\text{I}k\)\(\text{B}\)\(\alpha\) WT and ubiquitin-\(\text{I}k\)\(\text{B}\)\(\alpha\) fusion were expressed in HEK293 and stimulated for 20 minutes with 10 ng/ml of TNF\(\alpha\). D) \(\text{I}k\)\(\text{B}\)\(\alpha\) WT and ubiquitin-\(\text{I}k\)\(\text{B}\)\(\alpha\) fusion were expressed in HEK293 and stimulated for 20 minutes with 10 ng/ml of TNF\(\alpha\) for the indicated times.

doi:10.1371/journal.pone.0025397.g004

**Figure 5. Phosphorylation defect of ubiquitin-\(\text{I}k\)\(\text{B}\)\(\alpha\) fusion.** A) \(\text{I}k\)\(\text{B}\)\(\alpha\) WT and ubiquitin-\(\text{I}k\)\(\text{B}\)\(\alpha\) fusion were expressed in HEK293 cells and pretreated or not with 20 \(\mu\)M of MG132 for 1 hour before being stimulated with 10 ng/ml of TNF\(\alpha\) for 20 minutes. Sam68 was used as a charge control. B) Ubiquitin-\(\text{I}k\)\(\text{B}\)\(\alpha\) fusion deficiently binds endogenous IKK\(\beta\) and the \(\beta\)TrCP. \(\text{I}k\)\(\text{B}\)\(\alpha\) WT and ubiquitin-\(\text{I}k\)\(\text{B}\)\(\alpha\) fusion were expressed in HEK293 cells, pretreated or not with 20 \(\mu\)M of MG132 for 1 hour and stimulated with 10 ng/ml of TNF\(\alpha\) for 20 minutes. Cell lysates were immunoprecipitated with HA antibody and analyzed by Western-blot using indicated antibodies.

doi:10.1371/journal.pone.0025397.g005
Monoubiquitylated IκBα negatively affects NF-κB activity

To investigate the effect of ubiquitin-IκBα fusion on the NF-κB activity, first we explored its capacity to bind NF-κB and to inhibit NF-κB/DNA binding. Interaction with the NF-κB subunit p65 was tested in HEK293 cells expressing or not exogenous p65 and ubiquitin-IκBα or IκBα WT as indicated in Figure 6A. Our results clearly indicate that p65 co-immunoprecipitates equally well with both ubiquitin-IκBα and IκBα WT. To explore the capacity of ubiquitin-IκBα fusion to inhibit NF-κB/DNA binding, electrophoretic mobility shift assay (EMSA) were performed using increasing amounts of recombinant IκBα fusions proteins (1: 0.05 μl, 2: 0.1 μl and 3: 0.5 μl) and p65 were incubated with a radioactive labeled NF-κB probe for EMSA studies [12]. Comp.: competition with a 100-fold excess of the same unlabeled oligonucleotide added to the binding assay before the 32P-labeled probe. The graph corresponds to the mean of three independent experiments. C) IκBα KO fibroblasts were co-transfected with IκBα WT or ubiquitin-IκBα fusion expressing plasmid and a NF-κB luciferase reporter (3 EnhConA-Luc). Luciferase activity was measured as previously described [17]. The graph corresponds to the mean of three independent experiments.

doi:10.1371/journal.pone.0025397.g006

Figure 6. Ubiquitin-IκBα fusion negatively affects TNF-induced NF-κB activity. A) Ubiquitin-IκBα fusion protein has the same capacity as unmodified IκBα to bind to NF-κB. HEK293 cells were transfected with the indicated plasmids. Lysates were submitted to anti-HA immunoprecipitation and Western blotted with the indicated antibodies B) Ubiquitin-IκBα fusion protein inhibits NF-κB/DNA binding as well as unmodified IκBα. Different amount of recombinant IκBα fusions proteins (1: 0.05 μl, 2: 0.1 μl and 3: 0.5 μl) and p65 were incubated with a radioactive labeled NF-κB probe for EMSA studies [12]. Comp.: competition with a 100-fold excess of the same unlabeled oligonucleotide added to the binding assay before the 32P-labeled probe. The graph corresponds to the mean of three independent experiments. C) IκBα KO fibroblasts were co-transfected with IκBα WT or ubiquitin-IκBα fusion expressing plasmid and a NF-κB luciferase reporter (3 EnhConA-Luc). Luciferase activity was measured as previously described [17]. The graph corresponds to the mean of three independent experiments.

doi:10.1371/journal.pone.0025397.g006
concentrations of both ubiquitin-I\(\kappa\)B\(a\) and I\(\kappa\)B\(a\) WT. As observed in Figure 6B, the capacity of ubiquitin-I\(\kappa\)B\(a\) fusion to inhibit NF-\(\kappa\)B/DNA interaction is similar to the one of I\(\kappa\)B\(a\) WT. To further explore the effect of ubiquitin-I\(\kappa\)B\(a\) on the NF-\(\kappa\)B activity after TNF\(\alpha\) stimulation, luciferase reporter assays were set up. To avoid interferences with endogenous I\(\kappa\)B\(a\) MEF coming from I\(\kappa\)B\(a\) KO mice were employed. The inhibitory effect of ubiquitin-I\(\kappa\)B\(a\) became statistically significant after 6 hours of TNF\(\alpha\) stimulation (Figure 6C). Thus, our results indicate that in the presence of ubiquitin-I\(\kappa\)B\(a\) fusion, activation of NF-\(\kappa\)B is negatively regulated after TNF\(\alpha\) stimulation. Altogether, our results suggest the existence of distinct populations of I\(\kappa\)B\(a\) molecules among which mono-ubiquitylated I\(\kappa\)B\(a\) offers resistance to TNF\(\alpha\) mediated degradation preserving a dormant pool of NF-\(\kappa\)B that is different to the one activated through the activation of this signaling pathway (Figure 7).

Discussion

While polyubiquitylation of proteins has been associated to the regulation of signaling cascades or protein degradation by the Ubiquitin Proteasome System, monoubiquitylation or multiple monoubiquitylation have diverse non-catabolic functions [18]. Major technical problems to separate the ubiquitylated pool of endogenous proteins from unmodified ones justify our limited knowledge of the post-modification events. The broad distribution of monoubiquitylated I\(\kappa\)B\(a\) in multiple organs and cell lines underlines the \textit{in vivo} importance of this pool of I\(\kappa\)B\(a\). Endogenous monoubiquitylated I\(\kappa\)B\(a\) is stable after TNF\(\alpha\) stimulation and does coexist with polyubiquitylated I\(\kappa\)B\(a\) under the same conditions. Therefore monoubiquitylated I\(\kappa\)B\(a\) does not appear to be a precursor of polyubiquitylated forms of this inhibitor molecule, although a dynamic equilibrium between these populations cannot be excluded. Monoubiquitylated I\(\kappa\)B\(a\) accumulated after proteasome inhibitor and TNF\(\alpha\) treatment can be the result of proofreading mechanism acting on polyubiquitylated I\(\kappa\)B\(a\). With the help of a DUB-resistant ubiquitin-I\(\kappa\)B\(a\) fusion, results presented here show that monoubiquitylated I\(\kappa\)B\(a\) has an impact on basal and TNF\(\alpha\)-induced NF-\(\kappa\)B transcription. It remains to be investigated the nature of the stimuli (if any) able to drive an efficient proteolysis of monoubiquitylated I\(\kappa\)B\(a\). In a similar way SUMO-1 was reported to regulate I\(\kappa\)B\(a\) stability and NF-\(\kappa\)B transcription [10]. However it is unclear if both I\(\kappa\)B\(a\) pools

![Figure 7. Integrated view of the time-dependent contribution of monoubiquitylated versus non-modified form of I\(\kappa\)B\(a\) in the control of its proteasomal degradation and regulation of NF-\(\kappa\)B activity.](https://doi.org/10.1371/journal.pone.0025397.g007)
cooperate with each other to regulate basal and/or signal mediated turnover. Several lines of evidence suggest that monoubiquitylated IκBz might adopt a structured/protected conformation. The first evidence is the difficulty to pull down endogenous monoubiquitylated IκBz with various monoclonal and polyclonal antibodies. Second, the ubiquitin-IκBz fusion is not efficiently recognized by the IKKβ subunit resulting in a limited phosphorylation and binding to βTrCP. Finally, the impact of the fused ubiquitin on the IκBz resistance to TNFα-induced proteolysis, suggest reduced capacity to interact/get access to the proteasome. Attachment of monoubiquitin onto IκBz perhaps occludes IKK binding sites or creates molecular interference with this kinase. Under these circumstances, βTrCP might have difficulties to polyubiquitylate poorly phosphorylated IκBz. The unstructured extremities of IκBz favor the ubiquitin-independent proteasomal degradation of this molecule [19] justifying the necessity to generate a stable pool of monoubiquitylated IκBz. If there is an ubiquitin-protein ligase different than the SCF-βTrCP complex, it has to be proven. However, one has to keep in mind that E3-independent monoubiquitylation has been reported for proteins containing ubiquitin-binding domains [20]. Recently, monoubiquitylation of Rpn10 subunit of the proteasome has been shown to adopt a closed conformation due to the intramolecular interaction with its ubiquitin interacting motif or UIM [21]. In the case of Rpn10, monoubiquitylation affects presentation of ubiquitylated proteins to the proteasome. However, if this occluding mechanism exists for monoubiquitylated IκBz there is no evidence of an ubiquitin-binding domain present on this inhibitor. Nonetheless, the active molecular dynamics reported for the NF-κB system even under basal conditions [22] [23] [24] justifies the existence of monoubiquitylated IκBz as a cellular reservoir to regulate basal as well as signal-mediated NF-κB activity. Knowing the resistance to proteolysis observed for monoubiquitylated IκBz, one can speculate that an artificial increase of this pool could let to a better control of immune and/or inflammatory responses in organisms that have been exposed to multiple and/or sequential stimuli activating NF-κB. Future work will elucidate the role of the different populations of IκBz in the optimal control of this critical transcription factor.

Materials and Methods

Animals

Ethics Statement. Experiments were approved by the respective institutional committees for animal care and handling. Adult male Sprague-Dawley rats were deeply anesthetized with chloral hydrate and some tissues and organs were extracted. These samples were washed with cold PBS, immediately frozen in liquid nitrogen and stored at −80°C.

Cell cultures

HEK293 and HeLa (ATCC) were grown in DMEM (Gibco); Jurkat cells (ATCC) in RPMI (Gibco), all supplemented with 10% FBS and antibiotics. HEK293 and HeLa were transfected using lipofectamine (Invitrogen). For measurement of transcriptional activity, MEF null IκBz (kindly given by David Baltimore) were co-transfected with a NF-κB-luciferase reporter plasmid (3-EnhConA) and plasmids expressing IκBz WT or the ubiquitin-IκBz fusion. Luciferase activity was measured as previously described [17]. To analyze the half-life of the different proteins, cells were treated with 50 μg/ml of cycloheximide (Sigma) during the indicated times. For stability experiments, cells were treated for 1 hour with 20 μM of MG132 (Calbiochem), stimulated for 30 minutes or the indicated times with 10 ng/ml of TNFα (R&D Systems). P65, IKKβ and βTrCP co-immunoprecipitation experiments were performed using Protein-G cross-linked with the HA antibody to immunoprecipitate exogenous IκBz WT or ubiquitin-IκBz fusion protein. In all cases, cells were lysed for 15 minutes on ice in 50 mM sodium fluoride, 5 mM tetra-sodium pyrophosphate, 10 mM beta-glyceropropophosphate, 1% Igepal CA-630, 2 mM EDTA, 20 mM Na3HPO4, 20 mM NaH2PO4, 1 mM Pefablock, 1.2 mg/ml Complete protease inhibitor cocktail (Roche).

PCR and cloning

Ubiquitin gene (accession numbers CAA44911) was used to generate IκBz fusion and cloned into BamH1/Not1 restriction sites of pCDNA3. The C-terminal glycine residues (GG) of ubiquitin were changed to alanine (AA) and lysine 21 and 22 of IκBz were mutated to alanine to avoid respectively the action of DUBs and additional attachment of moieties at the N-terminus of IκBz, using the following oligonucleotides: 5'-ctc cgt cta aca gta ggc gag cgg cta ctg gac gac-3' and 5'-gac ctc gag ctc gag gac cgg cta cta ctc gag gac cgc-3'. His6-Ubiquitin construct has been previously reported [13].

Purification of ubiquitylated proteins

Frozen tissues were triturated in liquid nitrogen and recovered in the previously reported lysis buffers [14], containing 200 μg of TUBES-HR23A (T) (Life Sensors) or Glutathione S-transferase GST (C). Lysates were clarified by centrifugation, and added to glutathione agarose beads (Sigma). Glutathione beads were eluted and bound material was submitted to Western-blot analysis or to IκBz (Cell Signaling) and ubiquitin (FK2, ENZO) immunoprecipitations. Hist-ubiquitylated proteins were purified using denaturing conditions and Ni2+ chromatography as previously described [13].

Western blotting

Immunodetections were performed with the following primary antibodies: mouse monoclonals SV5 (Serootec); HA (Covance); Ubiquitin P4D1 (Santa Cruz Biotechnology Inc), FK2 (ENZO); IκBz (Cell Signaling), IκBz 10B (kindly provided by RT Hay) and anti-phospho-IκBz (Cell Signaling) antibodies; rabbit polyclonals IκBz (C21) (Santa Cruz Biotechnology); p65 (Santa Cruz Biotechnology); IKKβ (Cell Signaling) and Sam68 (Santa Cruz Biotechnology) antibodies and goat polyclonal antibody βTrCP (Santa Cruz Biotechnology).

In vitro ubiquitylation assays

In vitro transcribed/translated IκBz (35S-Met-labelled or not when indicated) were incubated in a 15 μl reaction including an ATP regenerating system [25 mM Tris pH 7.5, 5 mM MgCl2, 2 mM ATP, 10 mM creatine phosphate (Sigma), 5 mM NaCl2, 5.5 U/ml of creatine kinase (Sigma) and 0.6 U/ml of inorganic pyrophosphatase (Sigma)], 10 μg of ubiquitin mutant (Ub KO) where all reactive lysine residues have been changed to arginine ubiquitin, 10 ng human E1 (Boimol), 500 ng UbchH5b (Boimol). After incubation at 30°C for 120 min the reaction was stopped with SDS Laemmli buffer containing β-mercaptoethanol, samples were fractionated by SDS-PAGE and the dried gels analysed by phosphorimaging.

Electrophoretic Mobility Shift Assays (EMSA)

Reactions were prepared in binding buffer containing 25 mM HEPES, 1 mM EDTA, 3.5 mM spermidine, 6 mM MgCl2, 100 mM NaCl, 0.15% Nonidet P-40, 10% glycerol, 10 mM...
Dithiothreitol, 1 mg/ml bovine serum albumin and 0.05 mg Poly dA/dT/dG, different amount of IkBz fusion proteins (1: 0.05 μl, 2: 0.1 μl and 3: 0.5 μl) and recombinant protein p65 and incubated at room temperature for 20 minutes. Finally, 10000 cpm of 32P-radiolabelled (polynucleotide kinase, Biolabs) double strand oligonucleotide probe containing the NF-kB binding site motif from the HIV type 1 enhancer (5'CTG GAC GGG GAT TTC CGA GAG GT-3') was added and the mixture was incubated at room temperature for 20 minutes. After electrophoresis, gels were dried and exposed to Amersham Hyperfilm MP at ~70°C. Specific binding was checked by competition with a 100-fold excess of the same unlabeled oligonucleotide added to the binding assay before the 32P-labeled probe.

Supporting Information

Figure S1 Immunoprecipitation using IkBz antibodies fail to pull down monoubiquitylated IkBz. HEK293 cells were treated or not for 1 hour with 20 μM of MG-132, lysed in the properly lysis buffer for 20 minutes, centrifuged, and the supernatant was incubated with cross-linked anti-IkBz (10B) antibody for 2 hours. After incubation, the samples were centrifuged, washed and prepared for Western blot analysis using IkBz antibody (Cell Signaling).

(TIF)

Figure S2 TUBE-captured monoubiquitylated IkBz fails to be immunoprecipitated using specific IkBz antibodies. HEK293 cells were treated or not, 1 hour with 20 μM of MG-132 and lysed in a buffer containing 100 μg of TUBE-HR23A or GST proteins. After lysis, samples were centrifuged and clarified supernatant incubated for 2 hours in the presence of glutathione agarose beads. Eluted samples were incubated for 2 hours with protein A cross-linked antibody anti-IkBz 10B or anti-IkBz C21 antibody (not shown). After incubation, samples were washed and prepared for Western blot analysis using IkBz antibody (Cell Signaling).

(TIF)

Acknowledgments

We apologize that many relevant articles could not be cited due to space limitation. We would like to thank David Baltimore and Ron Hay for the IkBz null MEFs and IkBz 10B antibody respectively. We thank Alberto Pérez-Samartin and Fernando Lopitz-Ortosa for their technical contribution on this project.

Author Contributions

Conceived and designed the experiments: VI, CR, MSR. Performed the experiments: VI, MSR, EDS-F, MT-R, FA, MC. Analyzed the data: VI, MSR, CR, FA, MC. Contributed reagents/materials/analysis tools: CM, CR. Wrote the paper: EDS-F VI, MRS.

References

1. Hayden MS, Ghosh S (2008) Shared principles in NF-kappaB signaling. Cell 132: 344–362.
2. Li H, Lin X (2008) Positive and negative signaling components involved in TNFalpha-induced NF-kappaB activation. Cytokine 41: 1–8.
3. Mahi AM, Miyamoto S (2007) SUMO and NF-kappaB ties. Cell Mol Life Sci 64: 1979–1986.
4. Lang V, Rodriguez MS (2008) Innate link between NF-kappaB activity and ubiquitin-like modifiers. Biochem Soc Trans 36: 853–857.
5. Liu S, Chen ZJ (2010) Expanding role of ubiquitination in NF-kappaB signaling. Cell Res.
6. Krappmann D, Scheidereit C (2005) A pervasive role of ubiquitin conjugation in activation and termination of IkappaB kinase pathways. EMBO Rep 6: 321–326.
7. Chen ZJ (2005) Ubiquitin signalling in the NF-kappaB pathway. Nat Cell Biol 7: 738–765.
8. Baldi I, Brown K, Franzoso G, Siebenlist U (1996) Critical role for lysines 21 and 22 in signal-induced, ubiquitin-mediated proteolysis of I kappa B-alpha. J Biol Chem 271: 376–379.
9. Karin M, Ben-Neriah Y (2000) Phosphorylation meets ubiquitination: the control of NF-kappaB activity. Annu Rev Immunol 18: 621–663.
10. Desterro JM, Rodriguez MS, Hay RT (1998) SUMO-1 modification of IkappaBalpha inhibits NF-kappaB activation. Mol Cell 2: 233–239.
11. Ghosh S, Baltimore D (1990) Activation in vitro of NF-kappaB by phosphorylation of its inhibitor I kappa B. Nature 344: 678–682.
12. Arenzana-Seisdedos F, Thompson J, Rodriguez MS, Bacherie F, Thomas D, et al. (1995) Inducible nuclear expression of newly synthesized I kappa B alpha negatively regulates DNA-binding and transcriptional activities of NF-kappaB. Mol Cell Biol 15: 2685–2696.
13. Rodriguez MS, Trounson J, Hay RT, Dargemont C (1999) Nuclear retention of IkappaBalpha protects it from signal-induced degradation and inhibits nuclear factor kappaB transcriptional activation. J Biol Chem 274: 9108–9115.
14. Hoyer R, Allot F, Lopitz-Ortosa F, Lang V, England P, et al. (2009) Efficient protection and isolation of ubiquitylated proteins using tandem ubiquitin-binding entities. EMBO Rep 10: 1250–1258.
15. Carter S, Vosonden KH (2000) p53-ubl fusions as models of ubiquitination, sumoylation and neddylation of p53. Cell Cycle 7: 2519–2528.
16. Scherer DC, Brockman JA, Chen Z, Maniatis T, Ballard DW (1995) Signal-induced degradation of I kappa B alpha requires site-specific ubiquitination. Proc Natl Acad Sci U S A 92: 11259–11263.
17. Rodriguez MS, Wright J, Thompson J, Thomas D, Baleux F, et al. (1996) Identification of lysine residues required for signal-induced ubiquitination and degradation of I kappa B-alpha in vivo. Oncogene 12: 2425–2435.
18. Salmena L, Pandolfo P (2007) Changing venues for tumour suppression: balancing destruction and localization by monoubiquitination. Nat Rev Cancer 7: 409–413.
19. Mathes E, Wang L, Komives E, Ghosh G (2010) Flexible regions within I{kappa}B{alpha} create the ubiquitin-independent degradation signal. J Biol Chem 285: 32927–32936.
20. Hoeffler D, Heckman CM, Wagner S, Rogov V, Dotsch V, et al. (2007) E3-independent monoubiquitination of ubiquitin-binding proteins. Mol Cell 26: 891–898.
21. Isasa M, Kato E, Kim W, Yugo V, Gonzalez S, et al. (2010) Monoubiquitination of RPN10 regulates substrate recruitment to the proteasome. Mol Cell 38: 733–745.
22. Nelson DE, Ihekwaba AE, Elliott M, Johnson JR, Gibney CA, et al. (2004) Oscillations in NF-kappaB signaling control the dynamics of gene expression. Science 306: 704–708.
23. Hoffmann A, Levchenko A, Scott ML, Baltimore D (2002) The IkappaB-NF-kappaB signaling pathway: the first 15 years. Annu Rev Biochem 71: 845–909.
24. Mathes E, Wang L, Komives E, Ghosh G (2010) Flexible regions within I{kappa}B{alpha} create the ubiquitin-independent degradation signal. J Biol Chem 285: 32927–32936.
25. Hoeffler D, Heckman CM, Wagner S, Rogov V, Dotsch V, et al. (2007) E3-independent monoubiquitination of ubiquitin-binding proteins. Mol Cell 26: 891–898.