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Delineating role of ubiquitination on nuclear factor-kappa B pathway by a computational modeling approach

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ABSTRACT

Mutant ubiquitin found in neurodegenerative diseases has been thought to hamper activation of transcription factor nuclear factor-kappa B (NF-κB) by inhibiting ubiquitin–proteasome system (UPS). It has been reported that ubiquitin also is involved in signal transduction in an UPS-independent manner. We used a modeling and simulation approach to delineate the roles of ubiquitin on NF-κB activation. Inhibition of proteasome complex increased maximal activation of IKK mainly by decreasing the UPS efficiency. On the contrary, mutant ubiquitin decreased maximal activity of IKK. Computational modeling showed that the inhibition effect of mutant ubiquitin is mainly attributed to decreased activity of UPS-independent function of ubiquitin. Collectively, our results suggest that mutant ubiquitin affects NF-κB activation in an UPS-independent manner.

Introduction

NF-κB is a major transcription factor regulating a variety of cellular processes including inflammation, development, and apoptosis. In resting state, inhibitory kappa B proteins (IκBs), mainly IκBα, keep NF-κB in cytosol as a form of NF-κB–IκB complex [1]. External stimuli such as tumor necrosis factor (TNF), lymphotoxin-β, and lipopolysaccharides converge on phosphorylation of IκB kinase (IKK) complexes to activate NF-κB. Phosphorylated IKK complexs, in turn, phosphorylate IκB proteins, which now can be rapidly ubiquitinated by $SCF^{\beta TrCP}$. Ubiquitinated IκB is degraded via ubiquitin–proteasome system (UPS), resulting in free NF-κB which enters into nucleus and induces expression of its target genes. IκBs are bona fide NF-κB responsive proteins, therefore constitute a negative feedback.

Ubiquitination is an indispensible post-translational modification for NF-κB activation [2,3]. Ubiquitin is a conserved protein of 76 amino acids. Polyubiquitin chain linked via internal 48th lysine to the next glysine residue at the C-terminus (K48pUB) is a well known tag for degradation by proteasome. Substrates tagged with K48pUB are to be degraded by the proteasome complex, and the polyubiquitin chain is cleaved into ubiquitins. Recent reports support the idea that there is another form of polyubiquitin chain whose role is not related to protein degradation [3]. For example, polyubiquitin chain linked through internal 63rd lysine (K63pUB) is required for TNF-induced NF-κB activation [4–6].

Receptor Interacting Protein 1 (RIP1), one of proteins recruited to TNF receptor 1 complex, should be ubiquitinlated by K63pUB to mediate TNF-induced activation of transforming growth factor activated kinase 1 (TAK1) [7], which is immediate upstream of IKK complexes [8]. In this case, K63pUB is thought to serve as a platform on which TAK1 phosphorylates IKK complexes. Since NF-κB plays vital roles for normal cell survival and proliferation, sophisticated regulation of its activation is needed for normal cellular physiology, and therefore there are several ways to check NF-κB activation including above-mentioned negative feedback by IκBs. The other mechanism is mediated by degradation of RIP through UPS. A20 deubiquitinates K63pUB from RIP and subsequently ubiquitinates K48pUB on RIP in the early time periods of TNF stimulation [9]. Thus, ubiquitin engages in NF-κB activation via two modes: one, as the K48 form of RIP and IκB isoform in an UPS-dependent manner; and the other, through K63 form polyubiquitination of RIP in an UPS-independent manner.

Mutant ubiquitin (UBB^{+1}) found in neurodegenerative diseases has been considered to hamper NF-κB activation by inhibiting proteasome activity [10–12]. Theoretically, mutant ubiquitin can also affect the UPS-independent function of ubiquitin. Since K63pUB on RIP is a positive regulator of NF-κB activation, effects of mutant ubiquitin on NF-κB activation is not straightforward. In this work, we constructed a model of NF-κB activation focusing on the roles of ubiquitin.

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Methods

With a model for activation of IKK complex by TNF-α we used procedures described in the previous report [13] to find parameters of the model. We used pre-defined features when comparing experimental results with simulation results.

Modeling

NF-κB activation pathway downstream to TNFR is well known. However, considering all known mechanisms and molecules in detail makes it very difficult to determine parameters. Krishna et al. organized the NF-κB activation pathway into three components and found that maximal activity of IKK is the most important factor for NF-κB activation [14]. Therefore, we simplified NF-κB activation from TNFR focusing on IKK activation, as shown in Fig. 1.

After TNF binding to its receptor, several cytosolic proteins such as TRADD, RIP, and TRAF2 are recruited to TNFR to form TNFR complex [15,16]. K63pUB is conjugated to RIP in the complex, which in turn leads to TAK1 activation presumably by autophosphorylation. TAK1 is the major regulator of IKK activation in the signaling pathway from TNF to NF-κB activation [17]. Therefore, we regarded TNFR complex as inactive when it contains RIP without K63pUB. Conjugation of K63pUB to RIP in the TNFR complex, which is UPS-independent process, represents the activation of TNFR complex because the TNFR complex can induce IKK activation. A20 is an E3 ligase unique in that it can deubiquitinate K63pUB from RIP and ubiquitinate K48pUB on RIP, thereby, acts as a strong negative feedback mediator for NF-κB activation. It is unclear how TNF stimulation can induce the activity of A20 prior to its transcription by NF-κB [9]. We, therefore, incorporated this mechanism simply by adding activation mechanism of A20 by active TNFR complex. With this simplified model, we developed a series of ordinary differential equations in generalized mass action law as shown:

$$
\begin{aligned}
x_0' &= c_0 \\
x_1' &= a_{21}x_2 + a_{81}x_8 - a_{12}x_1x_0^u S \\
x_2' &= a_{12}x_1x_0^u S + a_{82}x_8 - a_{21}x_2 - a_{28}x_2x_3 \\
x_3' &= a_{43}x_4x_2 \\
x_4' &= -a_{43}x_4x_2 \\
x_5' &= a_{65}x_6 - a_{56}x_5x_2 \\
x_6' &= a_{56}x_5x_2 - a_{65}x_6 \\
x_7' &= 0 \\
x_8' &= a_{28}x_2x_3 - a_{82}x_8 - a_{81}x_8 - a_8x_0^{g_1}x_7^{h_1}
\end{aligned}
\tag{1}
$$

- Ubiquitin is assumed to be made constantly.
- Ubiquitin and proteasome have exponential term (u, g_1, h_1) less than 1. If exponential terms of ubiquitin and proteasome are 1, whole systems dynamics inevitably depends on the two molecules because they are in much higher concentration than other proteins. Ubiquitin has two exponential terms (u and g_1) because kinetics of TNFR inactivation and UPS activation are differentially regulated by ubiquitin.

Feature definition

Relevant parameters are not those of which simulation results exactly matched to those of experiment, but those of which simulation results showed similar dynamics to experimentally observed dynamics. Therefore we defined features for dynamics (activities along the time periods).

- T_{max}: The time at which activity is maximal.
- Half-time ($T_{1/2}$): The time at which activity is half of maximal activity.

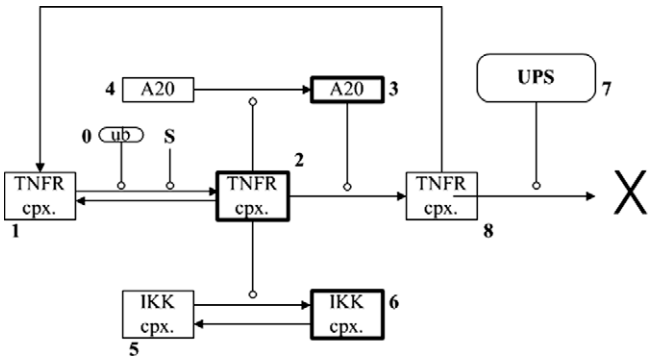

Fig. 1. Abridged model of IKK activation by TNF-α. Ligation of TNF-α to TNF receptor induces conformational change of cytosolic domain of TNF receptor, which can recruit several adaptor proteins such as TRADD, RIP, and TRAF2. Among them RIP and TRAF2 are conjugated with K63-linked polyubiquitin chain so as to activate IKK complex. The complex of TNF receptor and adaptor proteins is modelled to TNF complex (2 in Fig), which is activated by ubiquitin and signal (TNF-α). The active TNFR complex activates IKK complex and A20. A20 disassembles K63-linked polyubiquitin chain on RIP, therefore A20 is modelled to inactivate TNFR complex. A20 also conjugate K48-linked polyubiquitin on RIP, which leads to degradation of RIP via UPS. Molecules with bold frame denote active form.

- LVR (last-value ratio): ratio of maximal activity over activity at the last time.

For most cases, similarity of features can be used as similarity of overall dynamics. LVR indicates the time the signal terminates. We measured IKK activities at seven different time points, and interpolated the points to a function of Maxwell–Boltzmann distribution instead of spline function. All values of variables can be found by simple calculation with IKK activities. We extracted the features of the experimental data from the interpolated function.

Parameter selection

If randomly chosen parameters are close to real values measured in cells, simulation results with the parameters should reflect not only observed results under normal condition but also results obtained from cells perturbed. Therefore, we found parameters from normal condition and tested whether perturbed condition can be predicted by simulation with the parameters.

Initial step. We have chosen parameters randomly from the interval [0.0001, 1] and simulated with the parameters. Extracted features were compared with those from experiments. If the features satisfied criterion (Table 1), we continued the next step.

Perturbation step. We obtained the experimental data with perturbed conditions. We have run the simulation with parameters selected in the initial step, but in this moment, with modulated parameter(s) so as to mimic perturbed condition. If simulation results of perturbed condition were compatible with those observed experimentally perturbed condition, we have accept the parameters.

Table 1
Feature selection criteria.

Feature[a]	Experimental data	Selection range[b]
T_{max} (min)	21	Between 15 and 30
half-time (min)	54	Between 40 and 60
LVR[c]	0.11	Below 0.05

[a] Features are defined with IKK activities.
[b] Features for simulation were determined to cover experimental data within the range broad enough to maintain specificity.
[c] We used 90 min as the time period when the signal terminates. Even though LVR of experimental data is 0.11, this is likely to be over-estimated in the quantification step.

Simulation

Simulations were performed with a program made in C++ using gcc compiler. Algorithm 5.7 was used to find numerical solution of a system of ordinary differential equations as described previously [18].

Initial concentrations were set nominally. Concentrations of ubiquitin and proteasome are much higher than those of other proteins [19]. Initial concentration of ubiquitin was set as 70,000 and 1000 for proteasome complex. The values were set as 100 for normal TNFR and inactive IKK, and 200 for inactive A20. There is no active molecule at initial step. Simulation range was [0, 200] with 20,000 intervals.

Sensitivity analysis

To find variables that can represent efficiencies of UPS-independent ubiquitin conjugation and UPS-dependent proteolysis, we performed sensitivity analysis for each process. We measured maximal IKK activities while changing values of two variables involved in the each process and then determined a variable that has the most critical effect on maximal IKK activity. After finding the variables, we measured maximal IKK activities while changing the variables from 20% to 200% of values determined in normal cells.

Cell culture and reagents

Primary human fetal astrocytes and CRT-MG astrocytoma cells were maintained, as previously described [20,21] . Human recombinant TNF-α was purchased from R&D Systems (Minneapolis, MN, USA). A proteasome inhibitor, MG-132, was purchased from Calbiochem (La Jolla, CA, USA).

Western blot analysis

Cell lysates were electrophoresed in 12% SDS–PAGE and then transferred to nitrocellulose and probed with antibodies. Antibodies specific for IKK, phospho-IKKα/β ($Ser^{176/180}$), IκBα, and phospho-IκBα ($Ser^{32/36}$) were purchased from Cell Signaling (Beverly, MA, USA).

UBB^{+1} stable expression

For generation of the pEGFP-UBB^{+1} construct, the UBB^{+1} open reading frame was amplified by PCR from the pTet-Splice-UB plasmid and cloned in the EcoRI and HindIII sites of the EGFP-N1 vector (Clonetech, Palo Alto, CA). Stable cell lines transfected with the pEGFP or pEGFP-UBB^{+1} were generated. CRT-MG cells were transfected by electroporation (Amaxa Biosystems, Cologen, Germany) according to manufacturer's instruction. Stable transfectants were grown in medium containing 0.5 mg/ml G-418 (Life Technologies, Carlsbad, CA, USA) and cloned. Stable clonal cells were sorted by flow cytometry based on GFP fluorescence intensity.

Results

Feature extraction

Features of experimental data and selection range of features for simulation results are shown in Table 1. Because we are interested in tendency not the exact dynamics, we regarded parameters as acceptable when simulation results were within selection ranges.

Parameter selection

Simulation was performed 100,000 times. 3345 parameter sets were selected at initial step and among the parameters only 13 parameter sets were selected at perturbation step. Thus, it is very likely that perturbation step significantly increases accuracies of parameters. To check if the 13 parameter sets were adequate, we used another perturbation. Maximal IKK activity triggered by TNF in cells stably expressing mutant ubiquitin was lower than in normal cells, and simulation results of all the 13 parameters showed same results (Fig. 2). It is remarkable that the filtered 13 parameter sets predict effect of mutant ubiquitin on IKK activation well because the effect of mutant ubiquitin on IKK activation had not been taken into account when selecting parameters. Furthermore, predicting result of perturbation seems difficult (in case of proteasome inhibitor, among 3345 only 13 parameter sets successfully predicted the effect of proteasome inhibitor on IKK activation). Thus, we are sure that we found accurate parameters by incorporating perturbed condition in finding parameters.

We checked the consistency of the order of each parameter over the 13 sets because dynamics depends mainly on degree, not the exact value of the parameters. Orders of the values are listed in

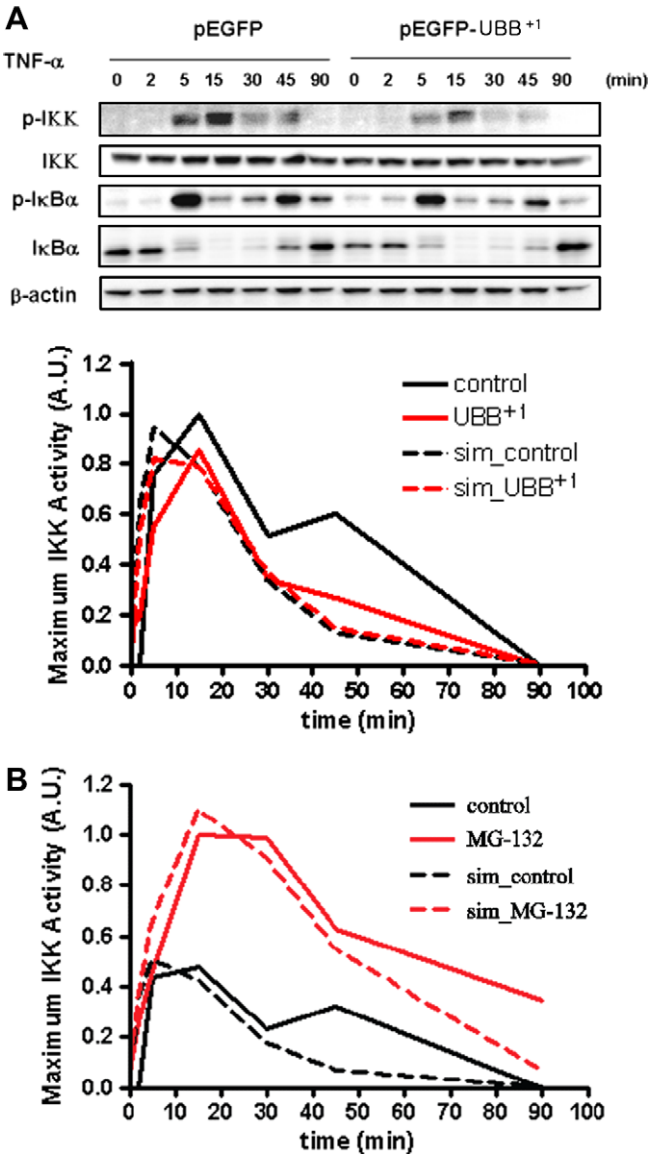

Fig. 2. IKK activity in cells having mutant ubiquitin (UBB^{+1}) and expected result with simulation. (A) Solid lines indicate quantification from experiment and dashed lines indicate expected activities by simulation. Decreased maximal IKK activity was observed in cells having mutant ubiquitin (upper panel), and this can be predicted from simulation (lower panel). (B) IKK activity in cells treated with MG-132 and expected results with simulation.

Supple Table 1. We can recognize from the table that relative sizes of reaction constants are consistent over all parameter sets. For example, reaction constant of IKK phosphorylation (a_{56}) is smaller than that of IKK dephosphorylation (a_{65}) in all parameter sets. Reaction constant for A20 activation in Supple Table 1 indicates that A20 activation is a very fast reaction (a_{43}, [22]). Those results were consistent with previously published results, implying that selected parameters represent physiological conditions well.

Differential effect of ubiquitin mutant and proteasome inhibition on IKK activation

There are two possible ways ubiquitin affects IKK activation. Degradation of RIP requires UPS-mediated proteolysis and has negative effect on IKK activation. On the other hand, conjugation on RIP of polyubiquitin chain linked by K63 is a typical UPS-independent process and has positive effect on IKK activity. Therefore, ubiquitin and proteasome complex are likely to have different roles in IKK activation. However, there is no experimental method to assess effects of ubiquitin while maintaining normal UPS activity. Therefore we used computational simulation to overcome the obstacle. From sensitivity analysis it has turned out that kinetic variable of proteasome concentration (h_1, Supple Fig. 1) and that of ubiquitin concentration (u, Supple Fig. 2) represent UPS efficiency and UPS-independent K63pUB-conjugation efficiency, respectively. Thus we measured maximal IKK activities while changing kinetic variables of proteasome and ubiquitin to mimic variations of UPS and K63pUB-conjugation efficiencies (Fig. 3).

It is clear that proteasome inhibitor and mutant ubiquitin affected maximal IKK activity in different ways to each other. It has been thought that mutant ubiquitin affect IKK activation by hampering UPS-dependent proteolysis. To our surprise, UPS efficiency was not significantly affected in cells expressing mutant ubiquitin since IκBα degradation in the cells was comparable to that in normal cells (Fig. 2). Therefore, it is thought that decreased maximal activity of IKK observed in cells stably expressing UBB^{+1} is attributed to decreased efficiency of UPS-independent processes (A in Fig. 3). On the other hand, increased maximal activity of IKK observed in cells treated with proteasome inhibitor may be caused mainly by decreased efficacy of UPS-dependent proteolysis (B in Fig. 3).

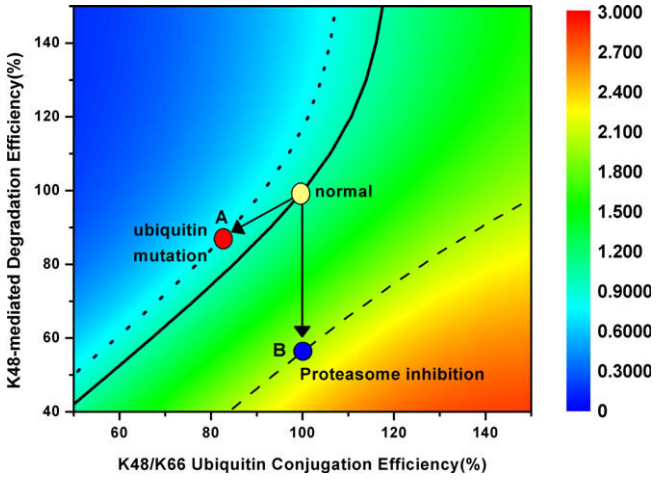

Fig. 3. Effects of ubiquitin conjugation and UPS on maximal IKK activity. Red closed circle indicates maximal activity of IKK in normal cells. Color represents maximal IKK activities on each condition. There is no change of maximal IKK level along the black thick line. Dotted line upper-left to thick black line indicates the parameter space where decreased maximal IKK activity is observed, and dashed line lower-right to thick black line means parameter space where increased maximal IKK activity is observed.

Discussion

It has been thought that mutant ubiquitin found in neurodegenerative diseases affects NF-κB activation by inhibiting proteasome activity, providing the rationale that proteasome inhibitors have been used to mimic effects of mutant ubiquitin (Ciechanover and Brundin, 2003). IKK complex is one of the most important molecules in NF-κB activation because several NF-κB activating signals converge on it. Ubiquitin involves in several steps of IKK activation in different ways. Conjugation of K63pUB on RIP is an UPS-independent process and has positive effect on IKK activation. On the other hand, degradation of RIP is an UPS-dependent process and has negative effect on IKK activation. Therefore mutant ubiquitin is likely to exert opposing effects on IKK activation simultaneously, which makes it difficult to appreciate the final effect of ubiquitin mutation on IKK activation. In this work, we built a model for IKK activation and tried to differentiate the effects of ubiquitin and proteasome on IKK activation by taking advantages of computational approach.

Finding values for parameters used in a model is a critical step in simulation. There are several methods proposed to surmount difficulty in parameter selection [23–26], but there are still difficulties in appreciating relevance of selected parameters. We incorporated perturbed condition in parameter finding step and obtained several sets of values. The values are consistent over all sets, thus, they are likely to be accurate. Furthermore, prediction made by simulation with the parameters matched well with experimental results. Next, we scrutinized different effects of mutant ubiquitin by UPS-dependent and UPS-independent manners on IKK activation pathway using simulation with the parameters.

We found that mutant ubiquitin decreases maximal IKK activity partly by decreasing UPS-dependent proteolysis, but mainly by decreasing UPS-independent K63pUB conjugation. On the other hand, proteasome inhibition by chemical inhibitors resulted in increased maximal IKK activity probably by stabilizing TRAF2 [27]. It is reasonable that proteasome inhibitors do not have any effects on UPS-independent ubiquitin conjugation. Therefore, effects of ubiquitin mutation and proteasome inhibition on IKK activationare quite different to each other. It implies that mutant ubiquitin found in neurological diseases has several effects on signal pathways other than just inhibition of the proteasome complex even though it hampers UPS in a dosage-dependent manner [28].

A frame-shift mutation of ubiquitin (UBB^{+1}) is frequently found in aging and AD brains, and is known to exert an inhibitory effect on the UPS. UBB^{+1} is generated by a transcriptional dinucleotide deletion within the mRNA, resulting in a 19-amino acid extension at the C-terminus [29]. UBB^{+1} cannot link to substrates targeted for proteasomal degradation but is itself ubiquitinated to form a poly-Ub chain. Poly-ubiquitinated UBB^{+1} is refractory to degradation by the proteasome complex and deubiquitinating enzymes, and thereby acts as a dominant negative form for both Lys-48-linked polyUb chain-dependent proteolysis as well as proteasome-independent functions such as Lys-63-linked polyUb chain-dependent signaling. It was reported that UBB^{+1} exerts a neurotoxic effect by suppressing proteasome-dependent proteolysis in neurons [10]. Although UBB^{+1} was also found in non-neuronal cells [30,31], its functional significance has not yet been determined.

To determine the effects of UBB^{+1} over-expression on UPS-mediated proinflammatory signaling in astrocytes, we examined the effects of proteasome inhibitors and UBB^{+1} over-expression on MKK activation, as it is an upstream signaling pathway involved in TNF-α-induced-AP-1 activation. Treatment with TNF-α-induced the phosphorylation and activation of MKK6 in a time-dependent manner. Preincubation with MG-132 markedly enhanced and pro-

longed the phosphorylation of MKK6 (Fig. 2). In stable cells transfected with the pEGFP control vector, treatment with TNF-α also induced the phosphorylation of MKK6 in a time-dependent manner. Unexpectedly, UBB^{+1} expression reduced TNF-α-induced phosphorylation of MKK. These conflicting results suggest that mutant ubiquitin affects the signaling pathway in a mainly proteasome-independent manner. The overall inhibition of UPS by UBB^{+1} over-expression results in decreased activation of proinflammatory signals and subsequent down-regulation of gene expression. It is possible that poly-ubiquitinated UBB^{+1}, which is refractory to deubiquitination, affects both Lys-48-linked polyUb chain-dependent proteolysis and Lys-63-linked polyUb chain-dependent signaling. Further studies are needed to delineate the modulatory effect of UBB^{+1} on UPS function and to identify the E3 ligases and DUBs of ubiquitinated UBB^{+1}.

Conclusion

In this study we found that effects of mutant ubiquitin on IKK activation differ from those of proteasome, but it is difficult to clarify roles of ubiquitin and proteasome on NF-κB activation because a model used in this study excluded many reactions in which ubiquitin and proteasome are involved. Even the limitation, conclusion that ubiquitin and proteasome have different roles on IKK complex activation indicates effects of mutant ubiquitin can be differ from those of malfunctioning UPS in several contexts other than IKK activation, and this distinction gets clear as UPS-independent function of ubiquitination are now increasing such as endocytosis and transcription.

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