

# Modeling the post-translational modifications and its effects in the NF- $\kappa$ B pathway

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## Abstract

The transcriptional activities of NF $\kappa$ B1 and NF $\kappa$ B2 complexes are regulated by several post-translational modifications at different sites of these complexes. The post-translational modifications reported in the literature include phosphorylation, methylation, acetylation, sulphhydration, nitrosylation, ubiquitination and sumoylation. We present a pathway network model with 172 proteins, 313 molecular species and 476 reactions including degradation of I $\kappa$ B (NF $\kappa$ BIA), proteolytic processing, and posttranslational modifications on p65 (RELA), RELB, p50 (NF $\kappa$ B1), and p52 (NF $\kappa$ B2) proteins. Comparing with experimental data on the over expression or knockouts of specific genes afforded qualitative agreement between model predictions and the experimental results. NF $\kappa$ B1:RELA complex is activated by active I $\kappa$ BKB, PIN1, MAP3K14 but repressed by PPARG and PDLIM2. MAP3K14 and UBE2I enhance the NF $\kappa$ B2:RELB activity, whereas NLRP12 is its repressor. The constitutive activation of NF $\kappa$ B1 complex through positive regulation of group of cytokines (IL1, IL1B and IL6) and IKK complex (alpha, beta and gamma) could be annulled by activation of PPARG, PIAS3 and P50-homodimer together instead of individual activations. Thus, the presented network pathway model has predictive utility in inferring NF- $\kappa$ B activity.

## Introduction

The Nuclear factor - kappa B (NF- $\kappa$ B) transcription factors regulate a wide range of cellular processes including inflammatory responses, cell differentiation and maturation of the immune system, secondary lymphoid organogenesis, stress responses, cell growth and development, and apoptosis (Gilmore 2006, Shih et al. 2011). These transcription factors are persistently active in several conditions such as chronic inflammation and cancer. There are two types of NF- $\kappa$ B signaling mediated by various mechanisms of post-translational modification and interactions with other molecules: canonical and non-canonical

(alternate) signaling pathway (Shih et al. 2011). Further, the NF- $\kappa$ B activities vary according to distinct modifications of the NF- $\kappa$ B subunits (Baeuerle and Baltimore, 1996; Li and Verma, 2002; Gilmore, 2006; Hayden and Ghosh, 2012; Christian *et al*, 2016; Zhang *et al*, 2017).

Originally identified as Nuclear Factor binding near the Kappa light-chain gene in B cells (Sen and Baltimore, 1986), NF- $\kappa$ B is ubiquitously present in all cell types. NF- $\kappa$ B proteins (homo- & hetero dimers/trimers of *NFKB1*, *RELA*, *NFKB2*, *RELB*, *REL*) are held in the cytoplasm in an inactive state by their interaction with members of the NF- $\kappa$ B inhibitor (I- $\kappa$ B) family (*NFKBIA*, *NFKBIB*, *NFKBID*, *NFKBIE*) each with its defined binding region (Jacobs and Harrison, 1998). In response to different stimuli and activators, inhibitor of kappa B (I- $\kappa$ B) is phosphorylated by I- $\kappa$ B kinase (IKK) and is consequently degraded, thus liberating the active NF- $\kappa$ B complex, which translocates to the nucleus (Sun, 2011; Smale, 2012; Christian *et al*, 2016). The activated NF- $\kappa$ B complex binding to specific  $\kappa$ B sites induces expression of varied classes of cytokines, chemokines and immune molecules (Gilmore 2006). It is now reliably established that depending on a variety of stimuli, NF-  $\kappa$ B complexes can trigger pro- or an anti-apoptotic pathway (Kaltschmidt *et al*, 2000; Baichwal and Baeuerle, 1997).

An approach to gain deeper understanding of functionalities of complex biological systems is the pathway network modeling (Kestler *et al*, 2008). Also, given the complexity of NF- $\kappa$ B pathway and its implication in disease mechanism, computational methods would enable the better understanding of signaling mechanism and activity regulation (Cheong *et al*, 2008). Early attempts to this end to probe the dynamics of NF- $\kappa$ B pathways through mathematical models are: temporal control of NF-kappaB activation by the coordinated degradation and synthesis of IkappaB proteins (I- $\kappa$ B alpha, I- $\kappa$ B beta, I- $\kappa$ B epsilon) (Hoffmann *et al*, 2002); negative feedback loop by I $\kappa$ B producing oscillations in NF- $\kappa$ B activity (Nelson *et al*, 2004); change in kinetics of NFKBIA, A20 and NF- $\kappa$ B activity (Lipniacki *et al*, 2004); oscillatory dynamics in NF- $\kappa$ B activity induced by p100 (Basak *et al*, 2007); negative feedback loop induced oscillation frequencies mediating the NF-kappaB-dependent gene expression (Ashall *et al*, 2009); pulsatile TNF stimulation providing distinct patterns of nonlinear NF- $\kappa$ B activation (Wang *et al*, 2011). These models have focused on the interplay between I- $\kappa$ B molecules, TNFAIP3, IKK complex, TNF and NF- $\kappa$ B complex, the related kinetics and the oscillating dynamics. Basak *et al*, 2012 have provided an account of different molecular components involved in the NF- $\kappa$ B signaling system and kinetic model for molecular interactions between the major components (I- $\kappa$ B, IKK, NEMO, NF- $\kappa$ B). The foremost attraction of their model is the rate constant parameters for association, dissociation, nuclear export/import of NF- $\kappa$ B, IKKcomplex, I- $\kappa$ B. These were derived either from literature or fitted through experimental data. Presently, several reports have been published in the literature on the post-translational modifications in the NF- $\kappa$ B pathway regulating its activity. Most of these connections and reactions were not available in the public databases, and therefore a manual curation from literature is

essential to build a comprehensive model using appropriate tool (Bauer-Mehren *et al*, 2009). We present here a NF- $\kappa$ B signaling network pathway model for both canonical and non-canonical pathways, incorporating core post-translational modifications on the proteins p65/RELA, RELB, p50/NFKB1, and p52/NFKB2. Our model describes the system behavior in the instances of activity of interacting molecular partners regulating NF- $\kappa$ B transcriptional activity by post-translational modifications. Our model allows systematic investigations of this pathway thereby explaining its functioning in comparison to experimental data.

## Result

### **The NF- $\kappa$ B complex network model simulation at default conditions exhibited transient kinetics for NFKB1 and persistent for NFKB2 activation, and empirical trends for experimental findings**

The canonical pathway is well studied compared to the non-canonical pathway. The former is activated by TNF and cytokines (IL1, IL1B, IL6) while the latter is majorly implicated in lymphoid organogenesis and activated by lymphotoxin beta receptor (LTBR) (Beinke, 2004; Lawrence, 2009; Liu *et al*, 2017). The integrated NF- $\kappa$ B complex activation network model representing the connections and reactions for the PTMs of NF- $\kappa$ B complex, IKK complex and other proteins (MAP3K14, TBK1, IKBKE, IRF1, IRF7, STAT1) was simulated for about 10-unit time to observe their effects on NF- $\kappa$ B activity.

Simulation of network model was carried out using mass-action kinetics. The results show that at initial default conditions where starting species were assigned a value of unity (Supplementary File, S1), the NFKB1 complex activation has rapid rise and fall representing transient kinetics. In contrast, the activation of NFKB2 followed a slow and persistent kinetic path (Sun, 2011; Fig. 3). The fall in NFKB1 activation accompanies RELA degradation and their connected relationship is displayed in Figure 3 (Saccani *et al*, 2004). Towards further verification of the performance of the network model, we carried out 10 cases of perturbations in the model for which, the experimental data are available and continued simulation for NF- $\kappa$ B activity. The results are summarized in Table 2 and Figures are presented in Supplementary File 2. The NF- $\kappa$ B activity is regulated both in the cytoplasm and the nucleus.

### **The transition of NFKB1 and NFKB2 complex from inactive to active state is not entirely dependent on NFKBIA (I- $\kappa$ B) degradation**

Post translational modifications namely, phosphorylation, ubiquitination, methylation, and acetylation impart their regulatory effect on transition of NF- $\kappa$ B complex to active state (Karin and Ben-Neriah, 2000; Supplementary File 2; Table 1, 2). Phosphorylation of RELA at specific Serine, Threonine or

Tyrosine residues by IKBKB, PRKACA, PRK CZ, TBK1, IKBKE, PIM1, RPS6KA1, RPS6KA4, RPS6KA5, CSNK2B, CSNK2A1, CSNK1D, SYK and PIN1 induced kinase, positively regulate the active state of NFKB1 complex (Christian *et al*, 2016; Table 1). Further, PRMT5 and NSD1 are methylases that methylate specific RELA-lysine residues thereby positively regulating the active state of NFKB1 complex (Lu *et al*, 2010; Table 1). The O-GlcNAcylation of T352-RELA induced by hyperglycemia (Yang *et al*, 2008), acetylation by CREBBP:EP300 complex at K310-RELA K314-RELA, K315-RELA, and acetylation by EP300 at K314-RELA also positively regulate the active state of NFKB1 complex (Chen *et al*, 2005; Buerki *et al*, 2008; Table 1). In the case of non-canonical pathway, CHUK, MAP3K14 and GSK3A positively regulate the active state of NFKB2 complex. In order to query the critical nodes, we carried out the over-expression and knockout of each of these proteins followed by simulation to identify the effect on regulation of active state of NF- $\kappa$ B complex.

We observed that in addition to over-expressed IKKcomplex (species 29 in the model) that sharply increases the active state of NFKB1 complex, proteins that are capable of modifying RELA irrespective of whether the inhibitor NFKBIA is bound or not, also contribute towards positive regulation of active state of NFKB1 complex. Setting over-expression of RPS6KA1 (species 1080), PIN1 (species 1191), MAP3K14 (species 1531) to 10 units (10 fold of default value), we observed that they positively regulated the active state of NFKB1 complex whereas, setting RPS6KA1, MAP3K14 to 0 units representing knockout conditions, had only marginal effect (Fig. 4). Because the over-expression of IKKcomplex has the intense positive regulatory effect on NFKB1 activity, We investigated the IKKcomplex knockdown by setting it to 0 units. This had marginal effect likely because other modifiers involved in phosphorylated-S536-RelA formation are still active and phosphorylated S536-RelA does not necessarily require the degradation of NFKBIA or p105/p50 for binding on promoter sites for NF- $\kappa$ B activation (Sasaki *et al*, 2005; Table 1; Fig. 2). The knockdown of PIN1 (setting PIN1 = 0 units) removed its stabilizing effect on RelA resulting in its degradation by SOCS1 pathway, leading to decrease in the active state of NFKB1 complex. Both events TP53 induced RPS6KA1 phosphorylation of RELA at S536 (Bohuslav *et al*, 2004) and PIN1 induced phosphorylation at T254-RelA, inhibits RelA and NFKBIA interaction (Ryo *et al*, 2003) thereby increasing the NFKB1complex activity. MAP3K14 indirectly increases the active state of NFKB1 complex by phosphorylating IKKalpha, which in turn activates IKKbeta thereby resulting in RELA phosphorylation at S276 and S536 by a number of kinases (Table 1); phosphorylated RelA undergoes acetylation at S310, which further increases the active state of NFKB1 complex. Thus, the NFKB1 related NF- $\kappa$ B activation alternately to I- $\kappa$ B degradation also is noteworthy contributor towards increased active state of NFKB1.

In the case of non-canonical pathway, over-expression of MAP3K14 (setting to 10 units) reveals that

MAP3K14, which is involved in processing of p100 and forming active p52:RELB, increases the active state of NFKB2 complex (Xiao *et al*, 2001; Xiao *et al*, 2004). UBE2I catalyzes sumoylation at K90, K298, K689, and K863 residues of p100 and the sumoylated p100 is primed for further proteosomal processing to form p52:RelB dimers. Knockout of UBE2I (when set to 0 units) decreases the active state of NFKB2 complex (Vatsyayan *et al*, 2008). Thus, the NFKB2 complex activation involving p100 proteosomal processing is an important contributor towards increased NF- $\kappa$ B activity.

### **Stimulating the RelA and RelB degradation are important for NF- $\kappa$ B activity suppression**

NF- $\kappa$ B, being a key transcription factor in regulating expression of numerous genes, is tightly controlled (Ghosh, 1999). The inducible NFKBIA mediated RelA nuclear export is considered to be the major mechanism of inhibition of the NF- $\kappa$ B complex activity in the form of a negative feedback oscillatory loop (Arenzana-Seisdedos *et al*, 1995). In this work, we considered other mechanisms of NF- $\kappa$ B inhibition in conjunction with NFKBIA mediated inhibition. The kinases CHEK1, ATR and GSK3B reduce the NFKB1 complex activity by phosphorylating RELA at specific residues thereby inducing its degradation (Crawley *et al*, 2015; Rocha *et al*, 2005; Zhao and Piwnica-Worms, 2001; Buss *et al*, 2004). NSD1 increases the active state of NFKB1 complex by methylating K218, K221 residues of RelA whereas KDM2A has been shown to inhibit this process by demethylating the same residues (Lu *et al*, 2010); SETD7 has been shown to negatively regulate the active state of NFKB1 complex by methylating K314, K315 residues of RelA (Yang *et al*, 2009). Acetylation of K122, K123 residues of RelA by KAT2B and EP300 suppress NF- $\kappa$ B activity. EP300 acetylates RelA at K314, K315 residues, which in turn promote the expression of specific set of genes while repressing the expression of another specific set of genes (Buerki *et al*, 2008). Moreover, ubiquitination of RelA by PPARG at K28 residue (Huo *et al*, 2012), by SOCS1 (Ryo *et al*, 2003) and by PDLIM2 (Tanaka *et al*, 2007) cause RelA degradation leading to decrease in active state of NFKB1 complex. Finally, sumoylation at K122, K123 of RelA catalyzed by PIAS3 also leads to the reduced NF- $\kappa$ B activity (Liu *et al*, 2012).

On over-expression (setting them to 10 units), we observed that SOCS1 (species 1193), PPARG (species 2) and PDLIM2 (species 1466) induce RelA degradation through ubiquitination, whereas PIAS3 (species 503) sumoylates RelA thereby forming transcription repression complex (Garcia-Dominguez and Reyes, 2009) and negatively regulates the active state of NFKB1 complex (Fig. 5). It has been reported that phosphorylation of T254 residue of RelA inhibits its degradation (Ryo *et al*, 2003). SOCS1 binds proximal to T254 residue of RelA thereby inhibiting its phosphorylation and expedites ubiquitin-mediated proteolysis of RelA (Ryo *et al*, 2003; Christian *et al*, 2016). RelA sumoylation induced by TNF and catalyzed by PIAS3 leads to RelA degradation suggesting either



negative feedback loop or pro-apoptotic mechanism orchestrated by TNF (Jang *et al*, 2004). We further observed that PDLIM2 is an important anti-inflammatory protein because it represses NF- $\kappa$ B activity by promoting RelA polyubiquitination and its subsequent degradation (Tanaka *et al*, 2007). PDLIM2 also aids in proteosomal degradation of STAT proteins thereby reducing activities of STAT1 and STAT4 (Tanaka *et al*, 2005). PPARG is unique in its action that it does not require NFKBIA removal and catalyzes K48-linked RelA polyubiquitination and its subsequent degradation (Huo *et al*, 2012). Thus, the mediators for RelA degradation through ubiquitination and mediators for NFKB1 transcription repression through sumoylation are direct repressors of NFKB1 related NF- $\kappa$ B activity.

In case of non-canonical pathway, GSK3B catalyzes phosphorylation of p100 (NFKB2) at S707, S711 residues thereby rendering p100 for ubiquitin mediated degradation by FBXW7 (Arabi *et al*, 2012). Also GSK3B catalyzes phosphorylation of RelB at S552 residue thereby stimulating its degradation (Neumann *et al*, 2012). Thus, GSK3B mediates NFKB2 degradation, which also functions as NFKBIA. RelB degradation is independent of degradation of NFKBIA. Further, NLRP12 activation mediates the MAP3K14 degradation thereby inhibiting the NFKB2 complex activity (Xiao *et al*, 2001; Williams *et al*, 2005; Lich *et al*, 2007). On over-expressing GSK3B (species 1098) and NLRP12 (species 1612) by setting to 10 units, it was observed that each negatively regulated the active state of NFKB2 complex. Therefore, NLRP12 and GSK3B have important roles for repressing NFKB2 related NF- $\kappa$ B activity.

### **PPARG, PIAS3 and P50 homodimer turn off the persistent activation of NF- $\kappa$ B**

It has been established that IKKcomplex induces the expression of proinflammatory cytokines and that IKKcomplex/NF- $\kappa$ B pathway leads to inflammation associated tumours and cancer development (Greten *et al*, 2004; Pikarsky *et al*, 2004). Positive regulation of activated IKKcomplex and high levels of proinflammatory cytokines lead to persistent activation of NF- $\kappa$ B, which may lead to tumour development, or to metabolic disorders (Chen *et al*, 2017).

The case of persistent activation of NF- $\kappa$ B was examined, by over-expressing IKKcomplex (species 29) and the group of cytokines (species 1068 encompassing IL1, IL1B, IL6 conglomerate) (setting to 10 units) each and simulated the model for 10 units time. A sharp increase in the active state of NFKB1 complex was observed (Fig. 6). After investigating for multiple negative regulators, it was inferred that any one regulator is not sufficient for complete repression of active state of NFKB1 complex. We had previously observed that over-expression of PIAS3 sharply decreases the active state of NFKB1 complex by inducing RelA sumoylation but it does not induce RelA degradation and the sumoylation event is dependent on NFKB1complex nuclear import. Because NF- $\kappa$ B can still show activity via other routes, therefore abolishing its activity also necessitate RelA degradation. Although it has been described that RelA degradation induced by PPARG is independent of NFKBIA removal from NFKB1

complex, PIAS3 supplementation with PPARG could not fully suppress the active state of NF $\kappa$ B1. The highest number of inhibitors of NF- $\kappa$ B signaling are NF- $\kappa$ B DNA binding inhibitors (Gilmore and Herscovitch, 2006). The p50 homodimer inhibits the NF- $\kappa$ B activity in default conditions (Kang *et al*, 1992; Plaksin *et al*, 1993). Thus, to achieve repressing the NF- $\kappa$ B *in silico* persistent activation, we set PIAS3 (species1 503), PPARG (species 2) and p50:p50 (species 1386) to 10 units each; and simulated the system for 10-unit time. This combination, as predicted, reduced the active state of NF- $\kappa$ B. Hence the functions of PPARG that induce RelA degradation and inhibition of insulin resistance, PIAS3 mediated RelA sumoylation and subsequent NF- $\kappa$ B activity repression, and p50 homodimer, which inhibits NF- $\kappa$ B DNA binding complement each other and reversed the effect of high levels of activated IKKcomplex and cytokines.

## Discussion

Our goal was to develop a pathway network model with all the post-translational modifications of NF- $\kappa$ B complex subunits for apprehending the differential regulation. Several assumptions were made in order to infer the dynamics of active state of of NF- $\kappa$ B complex using state transition modeling. Chiefly, we assume that the pool of enzymes catalyzing the PTMs is available in normal condition. Additionally, we have used a heuristic approach of assuming rate constants of unity and negative unity along with mass action kinetics. Therefore, the model developed here affords qualitative assessments with experimental results.

It has been suggested that targeting the processes that confer dysregulated immune response and cancer development, rather than the cell itself will be a better strategic therapeutic approach (Karin, 2008). A significant advancement in this work is to include reactions in the network model as a single system and explain NF- $\kappa$ B system behavior including both canonical and non-canonical pathways. Though the network model contains viral stimuli induced NF- $\kappa$ B activation, we haven't included its effect.

The complexities involved in the regulation of NF- $\kappa$ B activity were unraveled through transgenic and knockout mouse model studies (Gerondakis *et al*, 2006). In the canonical pathway, first, NFKBIA is phosphorylated, ubiquitinated and dissociates from RELA, followed by the phosphorylation of p105 and proteolytic cleavage to form p50: RELA complex, which then translocates to nucleus. In the case of non-canonical pathway, first p100 is phosphorylated and proteolytically cleaved to form p52, followed by NFKBIA phosphorylation, ubiquitination and dissociation from RELB to release p52: RELB, which then translocates to nucleus (Fig. 1a). However, translocation to nucleus does not necessarily initiate the transcription of NF- $\kappa$ B dependent gene expression, because there are reports that highlight the effect of PTMs in the nucleus on the NF- $\kappa$ B transcriptional activity regulation (Chen and Greene, 2004; Huang *et*



*al*, 2010). For example, there are evidences for NFKB1 activity even when NFKBIA is not phosphorylated and dissociated, and that some PTM events on RELA (p-316, p-S529, p-S536, O-GlcNAc-T352) are independent of NFKBIA phosphorylation and dissociation (Sasaki *et al*, 2005; Yang *et al*, 2008; Wang *et al*, 2015). Thus, there are two cellular compartmental levels of NF- $\kappa$ B activity regulation apparently independent of each other, namely, in cytoplasm and in nucleus. We included available evidence based connections between the modifications at CHUK, IKBKB, IKBKG, and activated IKKcomplex, and between post-translationally modified NF- $\kappa$ B complex and NF- $\kappa$ B activity, depicting their appropriate influence on NF- $\kappa$ B activity in the network model. Since the kinetic parameters for each PTM are not available in the literature, thus, it would be difficult to describe a system with known & unknown parameters. An assumption has been made that each reaction is simultaneous: similar rate constants each,  $k = +1$  for positive and  $k = -1$  for negative regulation on product species, and that initial reactants are ubiquitously present. Therefore, we have mapped the system behavior deemed to capture the totality of NF- $\kappa$ B activity regulation due to different post-translational modifications of NF- $\kappa$ B complex.

We used *in silico* over-expression or knockdown of the NF- $\kappa$ B system regulators for inferring the biological significance through numerical output. We procure heuristic trends through which, we were able to infer the differential effect of perturbations. We observed that MAP3K14 and PIN1 activation leading to increased NF- $\kappa$ B activity could be considered for potential therapeutics. In the case of hyper immune-activation circumstance, activation of PIAS3, PPARG and PDLIM2 could repress the NFKB1 complex activity; whereas activation of GSK3B and NLRP12 could reduce the NFKB2 complex activity. However, PIN1 activation could cause tumour development, and therefore the possibility of producing deleterious effects limits its candidature. Although MAP3K14 can also produce myeloma malignancies due to genetic abnormalities, yet as its expression and activity is tightly controlled, it can be a candidate for immune activation (Keats *et al*, 2007; Liao *et al*, 2004; He *et al*, 2006). PDLIM2 functions as nuclear E3 ubiquitin ligase in mediating RelA degradation through PML nuclear bodies (Tanaka *et al*, 2007), which imply essential NFKBIA removal and degradation steps. PDLIM2 role appears late in NF- $\kappa$ B activity regulation and so probably can also be the part of negative feedback mechanism. Nevertheless, its anti-inflammatory role is explicit and so qualifies as important negative regulator of NF- $\kappa$ B activity. Similar negative feedback loop is mediated through PIAS3 that induces NFKB1 complex suppression by sumoylating RelA at K122, K123 residues in the nucleus (Jang *et al*, 2004). Interestingly, EP300 and KAT2B acetylate the same residues and mediate NFKB1 complex activity inhibition through RelA nuclear export by NFKBIA, a reaction antagonized by HDAC3 that deacetylates Ac-K122, Ac-K123-RelA and increases NF- $\kappa$ B activity (Kiernan *et al*, 2003). On the other hand EP300 acetylates K314 residue of RelA, an event that increases the NF- $\kappa$ B activity (Buerki *et al*,

2008). EP300 in complex with CREBBP also results in increase in NF- $\kappa$ B activity by mediating K310-RelA acetylation and is involved in transcriptional activation of multiple processes (Chen *et al*, 2005; Tropberger *et al*, 2013). HDAC3 in association with IFRD1 deacetylate the Ac-K310-RelA to decrease NF- $\kappa$ B activity (Bakkar *et al*, 2008; Micheli *et al*, 2011). The acetylation event at K314-RelA by EP300 increases NF- $\kappa$ B activity and is competed by SETD7 that catalyzes methylation at the same residue leading to RelA proteosomal degradation and consequently decreasing NFKB1 complex activity (Yang *et al*, 2009). PIAS1 also appears as promising candidate for exhibiting its anti-inflammatory functions: (1) once activated by phosphorylation at S90 residue catalyzed by CHUK on TNF stimulation, PIAS1 reduces NFKB1 dependent NF- $\kappa$ B activity by inducing RelA sumoylation (Liu *et al*, 2013); (2) PIAS1 induces K703-STAT1 sumoylation, inhibiting STAT1 homodimer formation thereby reducing antiviral response (Ungureanu *et al*, 2003); (3) On TNF stimulation, MAPKAPK2-MAPK14 axis induced S522-PIAS1 phosphorylation increases the transrepression activity on NF- $\kappa$ B (Heo *et al*, 2013). While being anti-inflammatory, PIAS1 also inhibits p53 by sumoylating it, thereby inhibiting apoptosis and enforcing cell survival (Schmidt and Müller, 2002). PIAS1 inhibits CEBPB through sumoylation repressing adipogenesis and causing insulin resistance (Liu *et al*, 2013; Guo *et al*, 2015; Gustafson *et al*, 2015). Therefore caution is to be exercised for the exploratory usage of PIAS1. Yet another PIAS protein, PIAS4, is involved in sumoylating NEMO thus activating it and enhancing NF- $\kappa$ B activity in response to genotoxic agents (Mabb *et al*, 2006). Overall, the functions of SETD7, KAT2B and PIAS3 can be complemented to achieve better reduction in NFKB1 related NF- $\kappa$ B activity. Further, PPARG has been shown to be anti-inflammatory being the target of NSAIDs (Moon *et al*, 2014). Because PPARG induced RelA degradation is independent of its transcriptional activity (Huo *et al*, 2012) and NFKBIA sequestered NF- $\kappa$ B complex resides in cytoplasm (Arenzana-Seisdedos *et al*, 1995), therefore RelA degradation by PPARG is apparently independent of NFKBIA removal and hence of IKKcomplex activity. This is further supported by reports on efficient usage of PPARG agonists for the treatment of Type 2 diabetes and cancer (Sarraf *et al*, 1999; Panigrahy *et al*, 2002; Hausenblas *et al*, 2015). Also PPARG attenuates the PRKCA membrane translocation, which subsequently activates NF- $\kappa$ B, thus indirectly inhibiting the NF- $\kappa$ B activity (von Knethen *et al*, 2007).

With respect to non-canonical NF- $\kappa$ B signaling, the activation of GSK3B and NLRP12 could repress the NFKB2 related NF- $\kappa$ B activity. GSK3B mediated S468-RelA phosphorylation triggers RelA degradation (Buss *et al*, 2004), IKBKG phosphorylation at S17 and S31 activates IKKcomplex (Medunjanin *et al*, 2006). Moreover, GSK3B mediates the p105 (NFKB1) phosphorylation at S903 and S907 facilitating NF- $\kappa$ B function (Demarchi *et al*, 2003). GSK3B is additionally involved in inhibiting TNFSF10 mediated inhibition of prostate and pancreatic cancer cells (Liao *et al*, 2003; Mamaghani *et al*, 2012). Thus the ambivalent nature of GSK3B in canonical pathway limits its candidature and

therefore only NLRP12 succeeds as NFKB2 related NF- $\kappa$ B activity suppressor.

There has been significant research for the inhibitors of NF- $\kappa$ B signaling system and multiple molecules and therapies have been suggested, and are still in exploration (Gilmore and Herscovitch, 2006; Durand and Baldwin, 2017). However, the failure of the historic prime target of NF- $\kappa$ B signaling, IKBKB (Inhibitor of kappaB kinase beta) necessitates the identification of target and strategies that are less toxic and more profound (Prescott and Cook, 2018). We also observed that the knockdown of activated IKKcomplex does not inhibit the NF- $\kappa$ B activity (Fig. 6b) and this can be attributed towards proteins that directly mediate NF- $\kappa$ B activity by PTM and do not require NFKBIA degradation, another possible reason for IKBKB inhibitor failures. Therefore, to suppress the *insilico* model of NF- $\kappa$ B constitutive activation, we exploited the natural mechanism of NF- $\kappa$ B activity inhibition by increasing the level of PIAS3 that mediates the negative feedback inhibition, by increasing the levels of p50 homodimers that would inhibit NF- $\kappa$ B DNA binding, and by increasing the levels of PPARG that induces RelA degradation and counter the deleterious effects of canonical NF- $\kappa$ B constitutive activation. The schematic for the constitutive activation and its inhibition is represented in Figures 7a & 7b.

Some questions still remain regarding IKKcomplex constituents involved in influencing the NF- $\kappa$ B activity: (i) Whether CHUK and IKBKB are standalone kinases or require their assembly with IKBKG at each instance? (Karin, 1999); (ii) Whether IKBKG phosphorylation or sumoylation or ubiquitination is required at every instance of its involvement? (Sebban *et al*, 2006; Shifera, 2010); (iii) Whether phosphorylated RELA in nucleus is accompanied with acetylation or methylation event for its binding to cognate promoters at each occurrence? Thus, on the basis of available evidences as per our knowledge, we created an up-to-date NF- $\kappa$ B pathway network model incorporating its PTMs. We propose the manipulation of NF- $\kappa$ B signaling system using the inhibition (NF- $\kappa$ B subunits degradation, p50 homodimer induced NF- $\kappa$ B DNA binding inhibition, NF- $\kappa$ B subunits nuclear export and other feedback mechanisms), thereby employing the robust nature of biological system in self-inhibition. The NF- $\kappa$ B signaling network pathway model can be updated further accordingly and would assist in such predictions.

## Conclusion

Through the presented NF- $\kappa$ B network model, we were able to reproduce experimental observation heuristically. The model can be explored for various predictive studies in relation to NF- $\kappa$ B system and could be utilized for further experimental analyses. Investigating the NF- $\kappa$ B signaling system giving PTMs their due importance is imperative for proper therapeutic judgments. The method of model development and simulation presented here can also be applied to other pathway network as well. The

future lies in building and describing a model, which integrates the ‘real’ -parameters, -concentrations and -all related reactions, to mimic authentic biological network system.

## Materials and Method

### Network Model development

The NF- $\kappa$ B signaling system consists of homodimers or heterodimers or trimers made from different combinations of NFKB1 p105, RELA, NFKB1 p50, RELB, NFKB2 p100, NFKB2 p52, REL (cRel), NFKBIA (Shih et al. 2011) and their modifications represented both as individual species and in complex forms. A summarized version is displayed in Figure 1a. Among these proteins, all except REL are ubiquitously expressed in all cells. Presently, the role of REL in canonical or non-canonical pathways is not clearly described. Therefore we have not included REL in this first version of the NF- $\kappa$ B model with post-translational modifications. We have only considered NFKBIA as the Inhibitor of kappaB in the model as it is most studied and well characterized (Jacobs and Harrison, 1998; Dejardin *et al*, 1999).

The PubMed search with the keyword “NF- $\kappa$ B”, “NFkB” and “Nuclear factor kappa B” resulted in 657829 research publications including 5303 reviews, till 31<sup>st</sup> July 2018, highlighting the extent of research in the area. Bulk of information was derived from already mentioned reviews, after ascertaining the investigation context in the cited research article. Towards obtaining holistic information of the interacting proteins and their effects we extracted literature evidence-based connections while building the pathway network. Our goal was to capture the known NF- $\kappa$ B pathway signaling flow. The network model development and simulation been prepared using CellDesigner inbuilt functions as described (Matsuoka *et al*, 2014). Using the process diagram editor CellDesigner (Ver 4.4) (Funahashi *et al*, 2003), an up-to-date NF- $\kappa$ B activation network in System Biology Mark-up Language (SBML Level2, Version 4) format has been developed (Hucka *et al*, 2003). Pathway connections of the components having HGNC approved symbol (Yates *et al*, 2016), were assembled using KEGG (Kanehisa and Goto, 2000) and reactome pathway database (Joshi-Tope G *et al*, 2005; Fabregat *et al*, 2015) (Fig.1a). In case of ambiguities, we preferred annotations in reactome pathways. The model includes the reactions for reported modifications for each participating proteins at different amino acid residues and the reactions to map their consequential differential activities (Table 1). The evidences of reactions have been incorporated with reference to PMID of the publication as the attribute ‘Notes’ for the given connection. The reactions are thus denoted either as ‘positively regulated’ or ‘negatively regulated’, or dissociation reactions (for Inhibitor of kappaB (NFKBIA) dissociation each from RELA and RELB) (Fig. 1b). In order to eliminate connection errors with reactants and products iterative improvements were carried out

through simulations. The network model file in SBML format contains the entities reactants and products connected through reactions accompanied with reaction parameters such as ‘math for the reactions’, ‘initial concentration for the species’, and ‘boundary conditions for the species’ and the reaction evidences with cited references.

### **Model Simulation**

Built models must be tested for its utility by carrying out simulations and comparing the results with experimental data. For each simulation, the initial values for the species appearing first in sequence were set to 1 unit with ‘TRUE’ boundary conditions, and the rest species were set to 0 unit with ‘FALSE’ boundary conditions (Supplementary file, S1). The TRUE boundary condition restricts the concentrations in the range 0-1 whereas the false boundary conditions allow unrestricted variations in the concentrations of the species. The principle of Mass-action kinetics has been employed for the reactions, namely, the amount of product species is directly proportional to the product of amounts of reactants and rate constant  $k$ , where we have assumed  $k = 1$  for positive regulation, and  $k = -1$  for negative regulation reactions. However as the reaction rates become available, they can be included in the model for better precision simulations. The SBML ODE Solver Library (SOSlib) of CellDesigner has been used for simulation. The result for each run of simulation was observed at 10-unit time since start, when the growth or decay of several species was visible. This pattern offers a more clear understanding of the system behavior. Trial simulations for shorter times or longer times were not informative in this regard.

The net end output readings for network model simulation were observed principally as *transcriptional activation of NF- $\kappa$ B* for both canonical and non-canonical pathways. This preference was based on the fact that transcriptional activation of NF- $\kappa$ B regulates multiple genes of the immune system including several cytokines, chemokines and adhesion molecules (Lenardo *et al*, 1989; <http://www.bu.edu/nf-kb/gene-resources/target-genes/>). Additional outputs of general biological relevance recorded included effects on tumor formation, apoptosis, degradation of RelA, degradation of RelB, degradation of I- $\kappa$ B, degradation of NIK, cell migration, cell adhesion, and cell death. The scope of this model is restricted to different post-translation modifications of NF- $\kappa$ B impacting regulation of its transcriptional activation and not the differential gene expression downstream.

### *Explanation of Network Model Simulation Run*

Our goal was to build the model for NF- $\kappa$ B activation using state transition reactions where in the post-translational modifiers are also represented using the Boolean operator AND. Thus we could investigate the over-expression or knockout of a given modifier in terms of the outputs recorded through

simulations as described above.

The assumption made here is that the state transition is directly dependent on the concentrations of the molecular species carrying out the transition. Further, we assume that the metabolite donors (ATP, NADP etc.) are in abundance, so the kinetics are directly dependent on the concentrations of the proteins carrying out these state transitions and the proteins undergoing the state transitions.

Default Simulation is run at initial values as described above.

Perturbation conditions:

1. *In silico* knock out of candidate protein: Amount = 0 units; Boundary condition = TRUE; Constant = TRUE.
2. *In silico* overexpression of candidate protein: Amount = 10 units; Boundary condition = TRUE; Constant = TRUE.

The following steps were carried out for simulating the model:

- Load the Model file into the CellDesigner environment
- Open the ‘Simulation – Control Panel’ window from the Menu bar
- Set the ‘End Time = 10’ and ‘Execute’ the program
- For deducing the effect of intervention, change the ‘Initial Quantity’ accordingly, setting ‘Boundary Condition = TRUE’ and ‘Constant = TRUE’
- Save the image for each simulation event

A sample example has been explained in the Supplementary File 1. The complete model was deposited in BioModels (Chelliah *et al*, 2015) and assigned the identifier: MODEL2001290001.

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## **Author Contributions**

AM has prepared the network pathway model and carried out simulations. AM and SR have interpreted the results and have written the manuscript.

## **Conflict of Interest**

Authors declare that they have no conflict of interest.



## Reference

- Arabi A, Ullah K, Branca RM, Johansson J, Bandarra D, Haneklaus M, Fu J, Ariès I, Nilsson P, Den Boer ML, Pokrovskaja K. Proteomic screen reveals Fbw7 as a modulator of the NF- $\kappa$ B pathway. *Nature communications*. 2012 Jul 31;3:976.
- Arenzana-Seisdedos F, Thompson J, Rodriguez MS, Bachelier F, Thomas D, Hay RT. Inducible nuclear expression of newly synthesized I kappa B alpha negatively regulates DNA-binding and transcriptional activities of NF-kappa B. *Molecular and cellular biology*. 1995 May 1;15(5):2689-96.
- Ashall L, Horton CA, Nelson DE, Paszek P, Harper CV, Sillitoe K, Ryan S, Spiller DG, Unitt JF, Broomhead DS, Kell DB. Pulsatile stimulation determines timing and specificity of NF- $\kappa$ B-dependent transcription. *Science*. 2009 Apr 10;324(5924):242-6.
- Baeuerle PA, Baltimore D. NF- $\kappa$ B: ten years after. *Cell*. 1996 Oct 4;87(1):13-20.
- Baichwal VR, Baeuerle PA. Apoptosis: activate NF- $\kappa$ B or die?. *Current Biology*. 1997 Feb 1;7(2):R94-6.
- Bakkar N, Wang J, Ladner KJ, Wang H, Dahlman JM, Carathers M, Acharyya S, Rudnicki MA, Hollenbach AD, Guttridge DC. IKK/NF- $\kappa$ B regulates skeletal myogenesis via a signaling switch to inhibit differentiation and promote mitochondrial biogenesis. *J Cell Biol*. 2008 Feb 25;180(4):787-802.
- Basak S, Behar M, Hoffmann A. Lessons from mathematically modeling the NF -  $\kappa$  B pathway. *Immunological reviews*. 2012 Mar;246(1):221-38.
- Basak S, Kim H, Kearns JD, Tergaonkar V, O'Dea E, Werner SL, Benedict CA, Ware CF, Ghosh G, Verma IM, Hoffmann A. A fourth I  $\kappa$  B protein within the NF-  $\kappa$  B signaling module. *Cell*. 2007 Jan 26;128(2):369-81.
- Bauer-Mehren A, Furlong LI, Sanz F. Pathway databases and tools for their exploitation: benefits, current limitations and challenges. *Molecular systems biology*. 2009 Jan 1;5(1).
- Beinke S. Functions of NF- $\kappa$ B1 and NF- $\kappa$ B2 in immune cell biology. *Biochemical Journal*. 2004 Sep 1;382(2): 393-409.
- Bohuslav J, Chen LF, Kwon H, Mu Y, Greene WC. p53 induces NF- $\kappa$ B activation by an I $\kappa$ B kinase-independent mechanism involving phosphorylation of p65 by ribosomal S6 kinase 1. *Journal of Biological Chemistry*. 2004 Jun 18;279(25):26115-25.
- Buerki C, Rothgiesser KM, Valovka T, Owen HR, Rehrauer H, Fey M, Lane WS, Hottiger MO. Functional relevance of novel p300-mediated lysine 314 and 315 acetylation of RelA/p65. *Nucleic acids research*. 2008 Feb 7;36(5):1665-80.
- Buss H, Dörrie A, Schmitz ML, Frank R, Livingstone M, Resch K, Kracht M. Phosphorylation of serine 468 by GSK-3 $\beta$  negatively regulates basal p65 NF- $\kappa$ B activity. *Journal of Biological Chemistry*. 2004 Nov 26;279(48):49571-4.
- Chelliah V, Juty N, Ajmera I, Ali R, Dumousseau M, Glont M, Hucka M, Jalowicki G, Keating S, Knight-Schrijver V, Lloret-Villas A. BioModels: ten-year anniversary. *Nucleic acids research*. 2015 Jan 28;43(D1):D542-8.
- Chen LF, Greene WC. Shaping the nuclear action of NF- $\kappa$ B. *Nature reviews Molecular cell biology*. 2004 May;5(5):392.
- Chen LF, Williams SA, Mu Y, Nakano H, Duerr JM, Buckbinder L, Greene WC. NF- $\kappa$ B RelA phosphorylation regulates RelA acetylation. *Molecular and cellular biology*. 2005 Sep 15;25(18):7966-75.
- Chen Z, Yu R, Xiong Y, Du F, Zhu S. A vicious circle between insulin resistance and inflammation in nonalcoholic fatty liver disease. *Lipids in health and disease*. 2017 Dec;16(1):203.
- Cheong R, Hoffmann A, Levchenko A. Understanding NF- $\kappa$ B signaling via mathematical modeling. *Molecular systems biology*. 2008 Jan 1;4(1).
- Christian F, Smith EL, Carmody RJ. The regulation of NF- $\kappa$ B subunits by phosphorylation. *Cells*. 2016 Mar 18;5(1):12.

- Cohen P. The role of protein phosphorylation in human health and disease. *The FEBS Journal*. 2001 Oct 1;268(19):5001-10.
- Crawley CD, Kang S, Bernal GM, Wahlstrom JS, Voce DJ, Cahill KE, Garofalo A, Raleigh DR, Weichselbaum RR, Yamini B. S-phase-dependent p50/NF- $\kappa$ B1 phosphorylation in response to ATR and replication stress acts to maintain genomic stability. *Cell Cycle*. 2015 Feb 16;14(4):566-76.
- Dejardin E, Deregowski V, Chapelier M, Jacobs N, Gielen J, Merville MP, Bours V. Regulation of NF- $\kappa$ B activity by I $\kappa$ B-related proteins in adenocarcinoma cells. *Oncogene*. 1999 Apr;18(16): 2567.
- Demarchi F, Bertoli C, Sandy P, Schneider C. Glycogen synthase kinase-3 $\beta$  regulates NF- $\kappa$ B1/p105 stability. *Journal of Biological Chemistry*. 2003 Oct 10;278(41):39583-90.
- Durand JK, Baldwin AS. Targeting IKK and NF- $\kappa$ B for therapy. In *Advances in protein chemistry and structural biology 2017* Jan 1 (Vol. 107, pp. 77-115). Academic Press.
- Fabregat A, Sidiropoulos K, Garapati P, Gillespie M, Hausmann K, Haw R, Jassal B, Jupe S, Korfinger F, McKay S, Matthews L. The reactome pathway knowledgebase. *Nucleic acids research*. 2015 Dec 9;44(D1):D481-7.
- Funahashi A, Matsuoka Y, Jouraku A, Kitano H, Kikuchi N. CellDesigner: a modeling tool for biochemical networks. In *Proceedings of the 38th conference on Winter simulation 2006* Dec 3 (pp. 1707-1712). Winter Simulation Conference.
- Funahashi A, Morohashi M, Kitano H, Tanimura N. CellDesigner: a process diagram editor for gene-regulatory and biochemical networks. *Biosilico*. 2003 Nov 5;1(5):159-62.
- Gao N, Hibi Y, Cueno ME, Asamitsu K, Okamoto T. A-kinase interacting protein 1 (AKIP1) acts as a molecular determinant of the role of PKA in NF- $\kappa$ B signaling. *Journal of Biological Chemistry*. 2010 Jun 17;jbc-M110.
- Garcia-Dominguez M, Reyes JC. SUMO association with repressor complexes, emerging routes for transcriptional control. *Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms*. 2009 Jun 1;1789(6-8):451-9.
- Gerondakis S, Grumont R, Gugasyan R, Wong L, Isomura I, Ho W, Banerjee A. Unravelling the complexities of the NF- $\kappa$ B signalling pathway using mouse knockout and transgenic models. *Oncogene*. 2006 Oct;25(51):6781.
- Ghosh S. Regulation of inducible gene expression by the transcription factor NF- $\kappa$ B. *Immunologic research*. 1999 Jun 1;19(2-3):183-90.
- Gilmore TD, Herscovitch M. Inhibitors of NF- $\kappa$ B signaling: 785 and counting. *Oncogene*. 2006 Oct;25(51):6887.
- Gilmore TD. Introduction to NF- $\kappa$ B: players, pathways, perspectives. *Oncogene*. 2006 Oct 30;25(51):6680-4.
- Greten FR, Eckmann L, Greten TF, Park JM, Li ZW, Egan LJ, Kagnoff MF, Karin M. IKK $\beta$  links inflammation and tumorigenesis in a mouse model of colitis-associated cancer. *Cell*. 2004 Aug 6;118(3):285-96.
- Guo L, Li X, Tang QQ. Transcriptional regulation of adipocyte differentiation: a central role for CCAAT/enhancer-binding protein (C/EBP)  $\beta$ . *Journal of biological chemistry*. 2015 Jan 9;290(2):755-61.
- Gustafson B, Hedjazifar S, Gogg S, Hammarstedt A, Smith U. Insulin resistance and impaired adipogenesis. *Trends in Endocrinology & Metabolism*. 2015 Apr 1;26(4):193-200.
- Hausenblas HA, Schoulda JA, Smoliga JM. Resveratrol treatment as an adjunct to pharmacological management in type 2 diabetes mellitus—systematic review and meta-analysis. *Molecular nutrition & food research*. 2015 Jan;59(1):147-59.
- Hayden MS, Ghosh S. NF- $\kappa$ B, the first quarter-century: remarkable progress and outstanding questions. *Genes & development*. 2012 Feb 1;26(3):203-34.
- He JQ, Zarnegar B, Oganessian G, Saha SK, Yamazaki S, Doyle SE, Dempsey PW, Cheng G. Rescue of TRAF3-null mice by p100 NF- $\kappa$ B deficiency. *Journal of Experimental Medicine*. 2006 Oct 30;203(11):2413-8.

- Heo KS, Chang E, Takei Y, Le NT, Woo CH, Sullivan MA, Morrell C, Fujiwara K, Abe JI. Phosphorylation of PIAS1 by MAPK-activated protein kinase-2 inhibits endothelial inflammation via increasing both PIAS1 transrepression and SUMO E3 ligase activity. *Arteriosclerosis, thrombosis, and vascular biology*. 2013 Feb 1;33(2):321-9.
- Hoffmann A, Levchenko A, Scott ML, Baltimore D. The I $\kappa$ B-NF- $\kappa$ B signaling module: temporal control and selective gene activation. *Science*. 2002 Nov 8;298(5596):1241-5.
- Hou Y, Moreau F, Chadee K. PPAR $\gamma$  is an E3 ligase that induces the degradation of NF $\kappa$ B/p65. *Nature communications*. 2012 Dec 18;3:1300.
- Huang B, Yang XD, Lamb A, Chen LF. Posttranslational modifications of NF- $\kappa$ B: another layer of regulation for NF- $\kappa$ B signaling pathway. *Cellular signalling*. 2010 Sep 1;22(9):1282-90.
- Hucka M, Finney A, Sauro HM, Bolouri H, Doyle JC, Kitano H, Arkin AP, Bornstein BJ, Bray D, Cornish-Bowden A, Cuellar AA. The systems biology markup language (SBML): a medium for representation and exchange of biochemical network models. *Bioinformatics*. 2003 Mar 1;19(4):524-31.
- Jacobs MD, Harrison SC. Structure of an I $\kappa$ B $\alpha$ /NF- $\kappa$ B complex. *Cell*. 1998 Dec 11;95(6):749-58.
- Jang HD, Yoon K, Shin YJ, Kim J, Lee SY. PIAS3 suppresses NF- $\kappa$ B-mediated transcription by interacting with the p65/RelA subunit. *Journal of Biological Chemistry*. 2004 Jun 4;279(23):24873-80.
- Joshi-Tope G, Gillespie M, Vastrik I, D'Eustachio P, Schmidt E, de Bono B, Jassal B, Gopinath GR, Wu GR, Matthews L, Lewis S. Reactome: a knowledgebase of biological pathways. *Nucleic acids research*. 2005 Jan 1;33(suppl\_1):D428-32.
- Kaltschmidt B, Kaltschmidt C, Hofmann TG, Hehner SP, Dröge W, Schmitz ML. The pro-or anti-apoptotic function of NF- $\kappa$ B is determined by the nature of the apoptotic stimulus. *European Journal of Biochemistry*. 2000 Jun;267(12):3828-35.
- Kanehisa M, Goto S. KEGG: kyoto encyclopedia of genes and genomes. *Nucleic acids research*. 2000 Jan 1;28(1):27-30.
- Kang SM, Tran AC, Grilli M, Lenardo MJ. NF-kappa B subunit regulation in nontransformed CD4+ T lymphocytes. *Science*. 1992 Jun 5;256(5062):1452-6.
- Karin M, Ben-Neriah Y. Phosphorylation meets ubiquitination: the control of NF- $\kappa$ B activity. *Annual review of immunology*. 2000 Apr;18(1): 621-63.
- Karin M. How NF- $\kappa$ B is activated: the role of the I $\kappa$ B kinase (IKK) complex. *Oncogene*. 1999 Nov;18(49): 6867.
- Karin M. The I $\kappa$ B kinase—a bridge between inflammation and cancer. *Cell research*. 2008 Mar;18(3): 334.
- Keats JJ, Fonseca R, Chesi M, Schop R, Baker A, Chng WJ, Van Wier S, Tiedemann R, Shi CX, Sebag M, Braggio E. Promiscuous mutations activate the noncanonical NF- $\kappa$ B pathway in multiple myeloma. *Cancer cell*. 2007 Aug 14;12(2):131-44.
- Kestler HA, Wawra C, Kracher B, Köhl M. Network modeling of signal transduction: establishing the global view. *Bioessays*. 2008 Nov 1;30(11-12):1110-25.
- Kiernan R, Brès V, Ng RW, Coudart MP, El Messaoudi S, Sardet C, Jin DY, Emiliani S, Benkirane M. Post-activation turn-off of NF- $\kappa$ B-dependent transcription is regulated by acetylation of p65. *Journal of Biological Chemistry*. 2003 Jan 24;278(4):2758-66.
- Kitano H. Towards a theory of biological robustness. *Molecular systems biology*. 2007 Jan 1;3(1):137.
- Lawrence T. The nuclear factor NF- $\kappa$ B pathway in inflammation. *Cold Spring Harbor perspectives in biology*. 2009 Oct 7: a001651.
- Lenardo, M.J., Fan, C.M., Maniatis, T., and Baltimore, D. (1989). The involvement of NF-kappa B in beta-interferon gene regulation reveals its role as widely inducible mediator of signal transduction. *Cell* 57, 287–294.

- Li Q, Verma IM. NF- $\kappa$ B regulation in the immune system. *Nature Reviews Immunology*. 2002 Oct 1;2 (10):725-34.
- Liao G, Zhang M, Harhaj EW, Sun SC. Regulation of the NF- $\kappa$ B inducing kinase by TRAF3-induced degradation. *Journal of Biological Chemistry*. 2004 Apr 14.
- Liao X, Zhang L, Thrasher JB, Du J, Li B. Glycogen synthase kinase-3 $\beta$  suppression eliminates tumor necrosis factor-related apoptosis-inducing ligand resistance in prostate cancer. *Molecular cancer therapeutics*. 2003 Nov 1;2(11):1215-22.
- Lich JD, Williams KL, Moore CB, Arthur JC, Davis BK, Taxman DJ, Ting JP. Cutting edge: Monarch-1 suppresses non-canonical NF- $\kappa$ B activation and p52-dependent chemokine expression in monocytes. *The Journal of Immunology*. 2007 Feb 1;178(3):1256-60.
- Lipniacki T, Paszek P, Brasier AR, Luxon B, Kimmel M. Mathematical model of NF- $\kappa$ B regulatory module. *Journal of theoretical biology*. 2004 May 21;228(2):195-215.
- Liu T, Zhang L, Joo D, Sun SC. NF- $\kappa$ B signaling in inflammation. *Signal transduction and targeted therapy*. 2017 Jul 14;2: 17023.
- Liu Y, Bridges R, Wortham A, Kulesz-Martin M. NF- $\kappa$ B repression by PIAS3 mediated RelA SUMOylation. *PloS one*. 2012 May 23;7(5):e37636.
- Liu Y, Zhang YD, Guo L, Huang HY, Zhu H, Huang JX, Liu Y, Zhou SR, Dang YJ, Li X, Tang QQ. Protein Inhibitor of Activated STAT 1 (PIAS1) is Identified as the SUMO E3 Ligase of CCAAT/enhancer-binding protein (C/EBP)  $\beta$  during Adipogenesis. *Molecular and cellular biology*. 2013 Sep 23;MCB-00723.
- Lu T, Jackson MW, Wang B, Yang M, Chance MR, Miyagi M, Gudkov AV, Stark GR. Regulation of NF- $\kappa$ B by NSD1/FBXL11-dependent reversible lysine methylation of p65. *Proceedings of the National Academy of Sciences*. 2010 Jan 5;107(1):46-51.
- Mabb AM, Wuerzberger-Davis SM, Miyamoto S. PIASy mediates NEMO sumoylation and NF- $\kappa$ B activation in response to genotoxic stress. *Nature cell biology*. 2006 Sep;8(9):986.
- Mamaghani S, Simpson CD, Cao PM, Cheung M, Chow S, Bandarchi B, Schimmer AD, Hedley DW. Glycogen synthase kinase-3 inhibition sensitizes pancreatic cancer cells to TRAIL-induced apoptosis. *PLoS One*. 2012 Jul 19;7(7):e41102.
- Matsuoka Y, Funahashi A, Ghosh S, Kitano H. Modeling and simulation using CellDesigner. In: *Transcription Factor Regulatory Networks 2014* (pp. 121-145). Humana Press, New York, NY.
- Medunjanin S, Schleithoff L, Fiegehenn C, Weinert S, Zuschratter W, Braun-Dullaeus RC. GSK-3 $\beta$  controls NF- $\kappa$ B activity via IKK $\gamma$ /NEMO. *Scientific reports*. 2016 Dec 8;6:38553.
- Micheli L, Leonardi L, Conti F, Maresca G, Colazingari S, Mattei E, Lira SA, Farioli-Vecchioli S, Caruso M, Tirone F. PC4/Tis7/IFRD1 stimulates skeletal muscle regeneration and is involved in myoblast differentiation as a regulator of MyoD and NF- $\kappa$ B. *Journal of Biological Chemistry*. 2011 Feb 18;286(7):5691-707.
- Moon CM, Kwon JH, Kim JS, Oh SH, Jin Lee K, Park JJ, Pil Hong S, Cheon JH, Kim TI, Kim WH. Nonsteroidal anti-inflammatory drugs suppress cancer stem cells via inhibiting PTGS2 (cyclooxygenase 2) and NOTCH/HES1 and activating PPAR $\gamma$  in colorectal cancer. *International journal of cancer*. 2014 Feb 1;134(3):519-29.
- Nelson DE, Ihekwaba AE, Elliott M, Johnson JR, Gibney CA, Foreman BE, Nelson G, See V, Horton CA, Spiller DG, Edwards SW. Oscillations in NF- $\kappa$ B signaling control the dynamics of gene expression. *Science*. 2004 Oct 22;306(5696):704-8.
- Neumann M, Klar S, Wilisch-Neumann A, Hollenbach E, Kavuri S, Leverkus M, Kandolf R, Brunner-Weinzierl MC, Klingel K. Glycogen synthase kinase-3 $\beta$  is a crucial mediator of signal-induced RelB degradation. *Oncogene*. 2011 May;30(21):2485.
- Neves SR, Iyengar R. Modeling of signaling networks. *Bioessays*. 2002 Dec 1;24 (12):1110-7.
- Panigrahy D, Singer S, Shen LQ, Butterfield CE, Freedman DA, Chen EJ, Moses MA, Kilroy S, Duensing S, Fletcher C, Fletcher JA. PPAR $\gamma$  ligands inhibit primary tumor growth and metastasis by inhibiting angiogenesis. *The Journal of clinical investigation*. 2002 Oct 1;110(7):923-32.



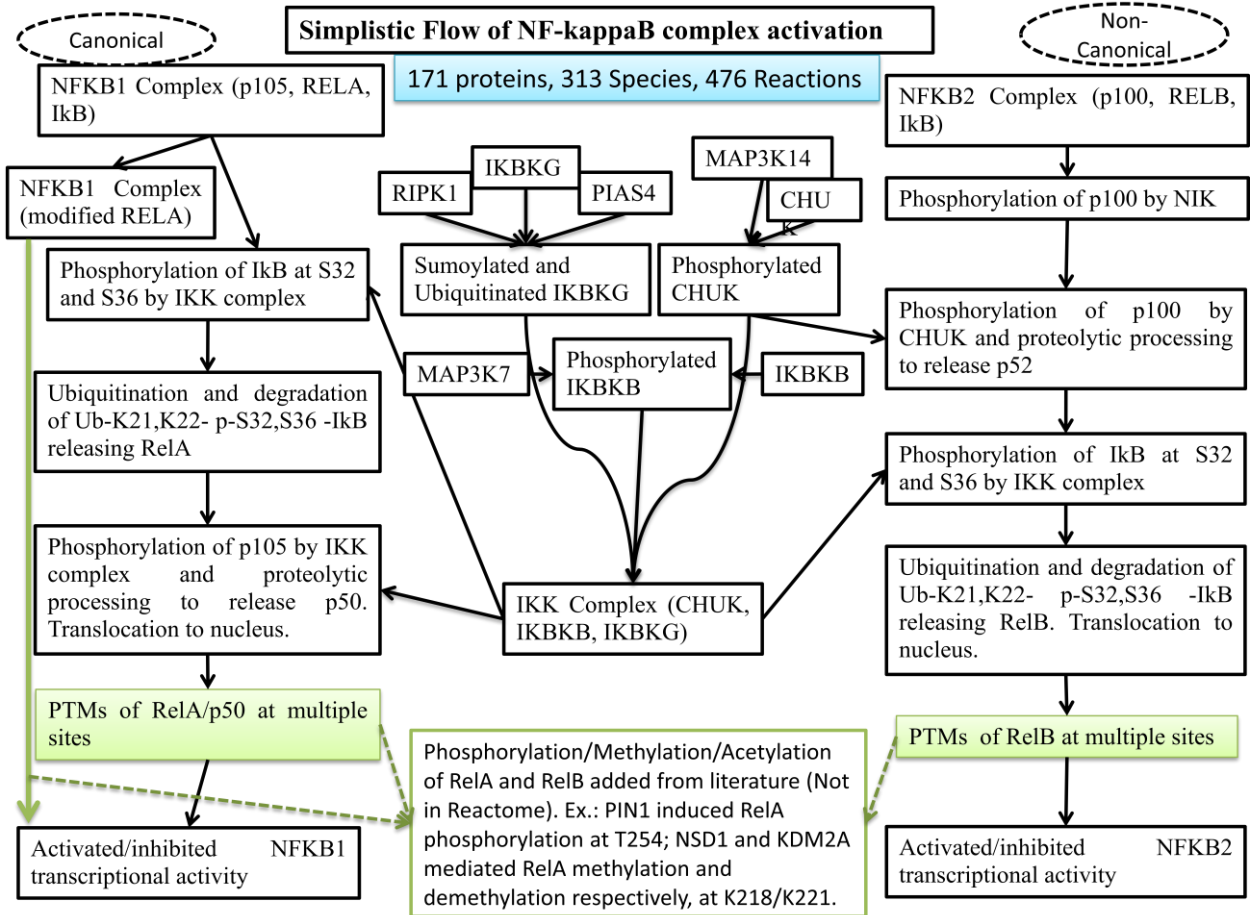
- Pikarsky E, Porat RM, Stein I, Abramovitch R, Amit S, Kasem S, Gutkovich-Pyest E, Urieli-Shoval S, Galun E, Ben-Neriah Y. NF- $\kappa$ B functions as a tumour promoter in inflammation-associated cancer. *Nature*. 2004 Sep;431(7007):461.
- Plaksin D, Baeuerle PA, Eisenbach L. KBF1 (p50 NF-kappa B homodimer) acts as a repressor of H-2Kb gene expression in metastatic tumor cells. *Journal of experimental Medicine*. 1993 Jun 1;177(6):1651-62.
- Prescott JA, Cook SJ. Targeting IKK $\beta$  in cancer: Challenges and opportunities for the therapeutic utilisation of IKK $\beta$  inhibitors. *Cells*. 2018 Sep;7(9):115.
- Rocha S, Garrett MD, Campbell KJ, Schumm K, Perkins ND. Regulation of NF- $\kappa$ B and p53 through activation of ATR and Chk1 by the ARF tumour suppressor. *The EMBO journal*. 2005 Mar 23;24(6):1157-69.
- Roy SK, Hu J, Meng Q, Xia Y, Shapiro PS, Reddy SP, Plataniias LC, Lindner DJ, Johnson PF, Pritchard C, Pagés G. MEKK1 plays a critical role in activating the transcription factor C/EBP- $\beta$ -dependent gene expression in response to IFN- $\gamma$ . *Proceedings of the National Academy of Sciences*. 2002 Jun 11;99(12):7945-50.
- Ryo A, Suizu F, Yoshida Y, Perrem K, Liou YC, Wulf G, Rottapel R, Yamaoka S, Lu KP. Regulation of NF- $\kappa$ B signaling by Pin1-dependent prolyl isomerization and ubiquitin-mediated proteolysis of p65/RelA. *Molecular cell*. 2003 Dec 1;12(6):1413-26.
- Saccani S, Marazzi I, Beg AA, Natoli G. Degradation of promoter-bound p65/RelA is essential for the prompt termination of the nuclear factor  $\kappa$ B response. *Journal of Experimental Medicine*. 2004 Jul 5;200(1):107-13.
- Sarraf P, Mueller E, Smith WM, Wright HM, Kum JB, Aaltonen LA, de la Chapelle A, Spiegelman BM, Eng C. Loss-of-function mutations in PPAR $\gamma$  associated with human colon cancer. *Molecular cell*. 1999 Jun 1;3(6):799-804.
- Sasaki CY, Barberi TJ, Ghosh P, Longo DL. Phosphorylation of RelA/p65 on serine 536 defines an I $\kappa$ B $\alpha$ -independent NF- $\kappa$ B pathway. *Journal of Biological chemistry*. 2005 Aug 16.
- Schmidt D, Müller S. Members of the PIAS family act as SUMO ligases for c-Jun and p53 and repress p53 activity. *Proceedings of the National Academy of Sciences*. 2002 Mar 5;99(5):2872-7.
- Sebban H, Yamaoka S, Courtois G. Posttranslational modifications of NEMO and its partners in NF- $\kappa$ B signaling. *Trends in cell biology*. 2006 Nov 1;16(11):569-77.
- Sen, R., and Baltimore, D. (1986). Multiple nuclear factors interact with the immunoglobulin enhancer sequences. *Cell* 46, 705–716.
- Shifera AS. Protein–protein interactions involving IKK $\gamma$  (NEMO) that promote the activation of NF- $\kappa$ B. *Journal of cellular physiology*. 2010 Jun 1;223(3): 558-61.
- Shih VF, Tsui R, Caldwell A, Hoffmann A. A single NF $\kappa$ B system for both canonical and non-canonical signaling. *Cell research*. 2011 Jan;21(1):86.
- Smale ST. Dimer-specific regulatory mechanisms within the NF- $\kappa$ B family of transcription factors. *Immunological reviews*. 2012 Mar 1;246(1):193-204.
- Sun SC. Non-canonical NF- $\kappa$ B signaling pathway. *Cell research*. 2011 Jan;21(1):71.
- Sun SC. The non-canonical NF- $\kappa$ B pathway in immunity and inflammation. *Nature reviews immunology*. 2017 Sep;17(9): 545.
- Tanaka T, Grusby MJ, Kaisho T. PDLIM2-mediated termination of transcription factor NF- $\kappa$ B activation by intranuclear sequestration and degradation of the p65 subunit. *Nature immunology*. 2007 Jun;8(6):584.
- Tanaka T, Soriano MA, Grusby MJ. SLIM is a nuclear ubiquitin E3 ligase that negatively regulates STAT signaling. *Immunity*. 2005 Jun 1;22(6):729-36.
- Tropberger P, Pott S, Keller C, Kamieniarz-Gdula K, Caron M, Richter F, Li G, Mittler G, Liu ET, Bühler M, Margueron R. Regulation of transcription through acetylation of H3K122 on the lateral surface of the histone octamer. *Cell*. 2013 Feb 14;152(4):859-72.

- Ungureanu D, Vanhatupa S, Kotaja N, Yang J, Aittomäki S, Jänne OA, Palvimo JJ, Silvennoinen O. PIAS proteins promote SUMO-1 conjugation to STAT1. *Blood*. 2003 Nov 1;102(9):3311-3.
- Valk E, Venta R, Örd M, Faustova I, Kõivomägi M, Loog M. Multistep phosphorylation systems: tunable components of biological signaling circuits. *Molecular biology of the cell*. 2014 Nov 5;25(22):3456-60.
- Vatsyayan J, Qing G, Xiao G, Hu J. SUMO1 modification of NF- $\kappa$ B2/p100 is essential for stimuli-induced p100 phosphorylation and processing. *EMBO reports*. 2008 Sep 1;9(9):885-90.
- von Knethen A, Soller M, Tzieply N, Weigert A, Johann AM, Jennewein C, Köhl R, Brüne B. PPAR $\gamma$ 1 attenuates cytosol to membrane translocation of PKC $\alpha$  to desensitize monocytes/macrophages. *The Journal of cell biology*. 2007 Feb 26;176(5):681-94.
- Wang B, Wei H, Prabhu L, Zhao W, Martin M, Hartley AV, Lu T. Role of novel serine 316 phosphorylation of the p65 subunit of NF- $\kappa$ B in differential gene regulation. *Journal of Biological Chemistry*. 2015 Jun 16;jbc-M115.
- Wang Y, Paszek P, Horton CA, Kell DB, White MR, Broomhead DS, Muldoon MR. Interactions among oscillatory pathways in NF-kappa B signaling. *BMC systems biology*. 2011 Dec;5(1):23.
- Williams KL, Lich JD, Duncan JA, Reed W, Rallabhandi P, Moore C, Kurtz S, Coffield VM, Accavitti-Loper MA, Su L, Vogel SN. The CATERPILLER protein Monarch-1 is an antagonist of Toll-like receptor-, tumor necrosis factor  $\alpha$ -, and Mycobacterium tuberculosis-induced pro-inflammatory signals. *Journal of Biological Chemistry*. 2005 Dec 2;280(48):39914-24.
- Xiao G, Fong A, Sun SC. Induction of p100 processing by NF- $\kappa$ B-inducing kinase involves docking I $\kappa$ B kinase  $\alpha$  (IKK $\alpha$ ) to p100 and IKK $\alpha$ -mediated phosphorylation. *Journal of Biological Chemistry*. 2004 Jul 16;279(29):30099-105.
- Xiao G, Harhaj EW, Sun SC. NF- $\kappa$ B-inducing kinase regulates the processing of NF- $\kappa$ B2 p100. *Molecular cell*. 2001 Feb 1;7(2):401-9.
- Yang WH, Park SY, Nam HW, Kang JG, Kang ES, Kim YS, Lee HC, Kim KS, Cho JW. NF $\kappa$ B activation is associated with its O-GlcNAcylation state under hyperglycemic conditions. *Proceedings of the National Academy of Sciences*. 2008 Nov 11;105(45):17345-50.
- Yang XD, Huang B, Li M, Lamb A, Kelleher NL, Chen LF. Negative regulation of NF- $\kappa$ B action by Set9-mediated lysine methylation of the RelA subunit. *The EMBO journal*. 2009 Apr 22;28(8):1055-66.
- Yates B, Braschi B, Gray KA, Seal RL, Tweedie S, Bruford EA. Genenames.org: the HGNC and VGNC resources in 2017. *Nucleic acids research*. 2016 Oct 30;gkw1033.
- Zhang Q, Lenardo MJ, Baltimore D. 30 Years of NF- $\kappa$ B: a blossoming of relevance to human pathobiology. *cell*. 2017 Jan 12;168(1):37-57.
- Zhao HU, Piwnica-Worms H. ATR-mediated checkpoint pathways regulate phosphorylation and activation of human Chk1. *Molecular and cellular biology*. 2001 Jul 1;21(13):4129-39.
- Zhong H, Voll RE, Ghosh S. Phosphorylation of NF- $\kappa$ B p65 by PKA stimulates transcriptional activity by promoting a novel bivalent interaction with the coactivator CBP/p300. *Molecular cell*. 1998 Apr 1;1(5):661-71.

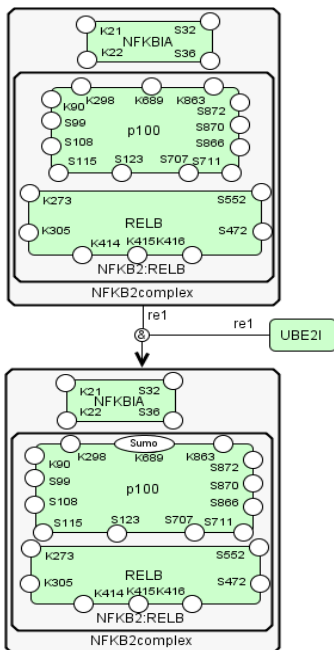
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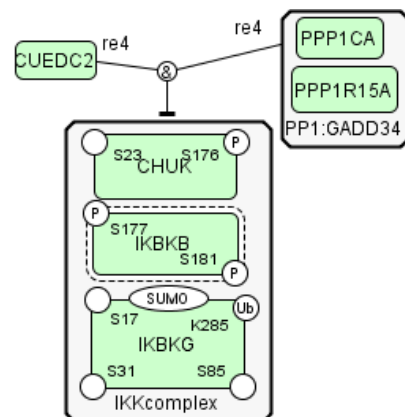
a.

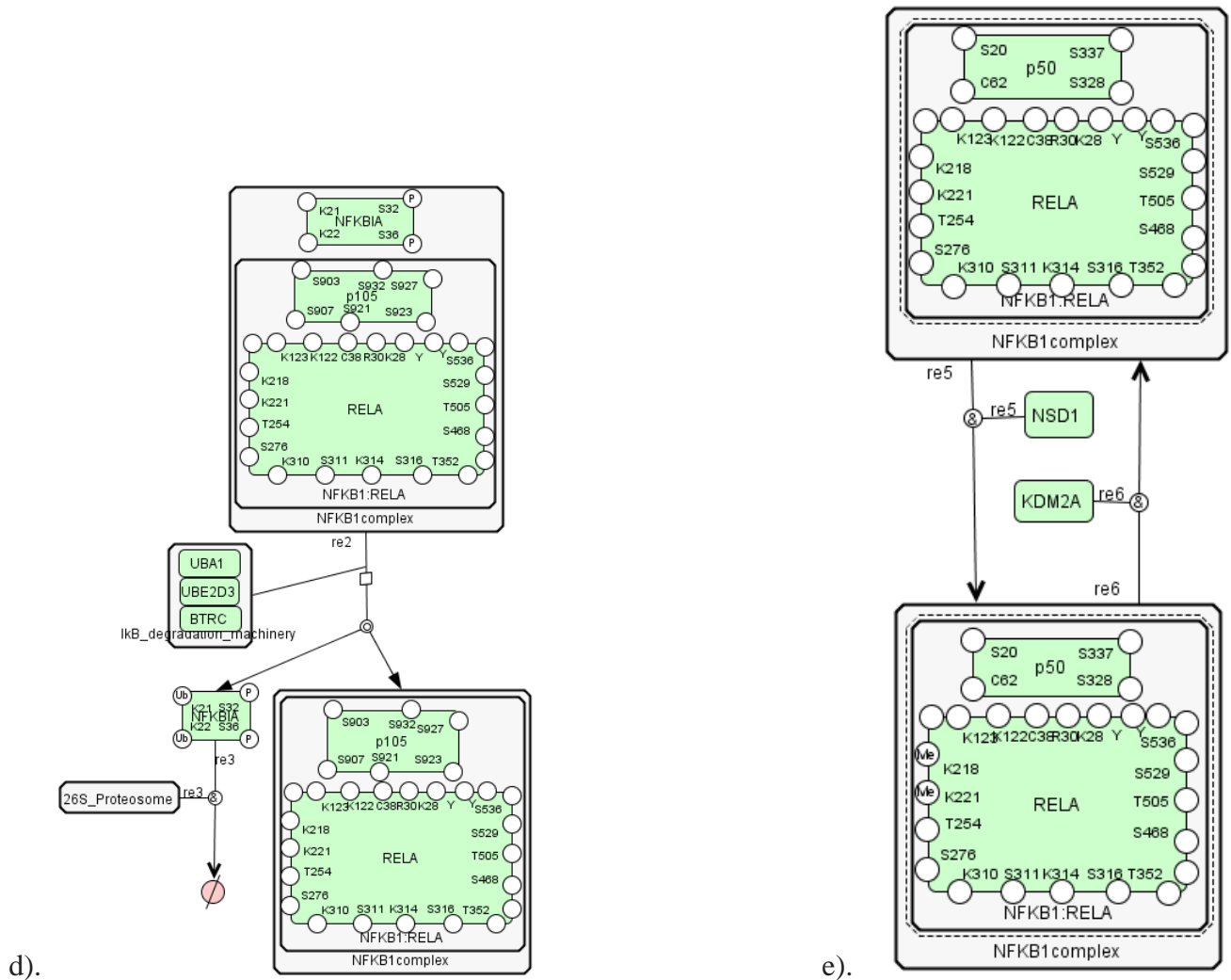


b.



c.





**Figure 1:** Network model description.

**a.** The NF-κB system pathway flow: canonical (NFKB1:RELA) and non-canonical/alternate pathway (NFKB2:RELB). It includes the post-translational modification of RelA, RelB, p105 (and consequent processing to p50), p100 (and consequent processing to p52), and their effect on the NFKB1 and NFKB2 related transcriptional activity. The detailed list of PTMs is displayed in Table 1.

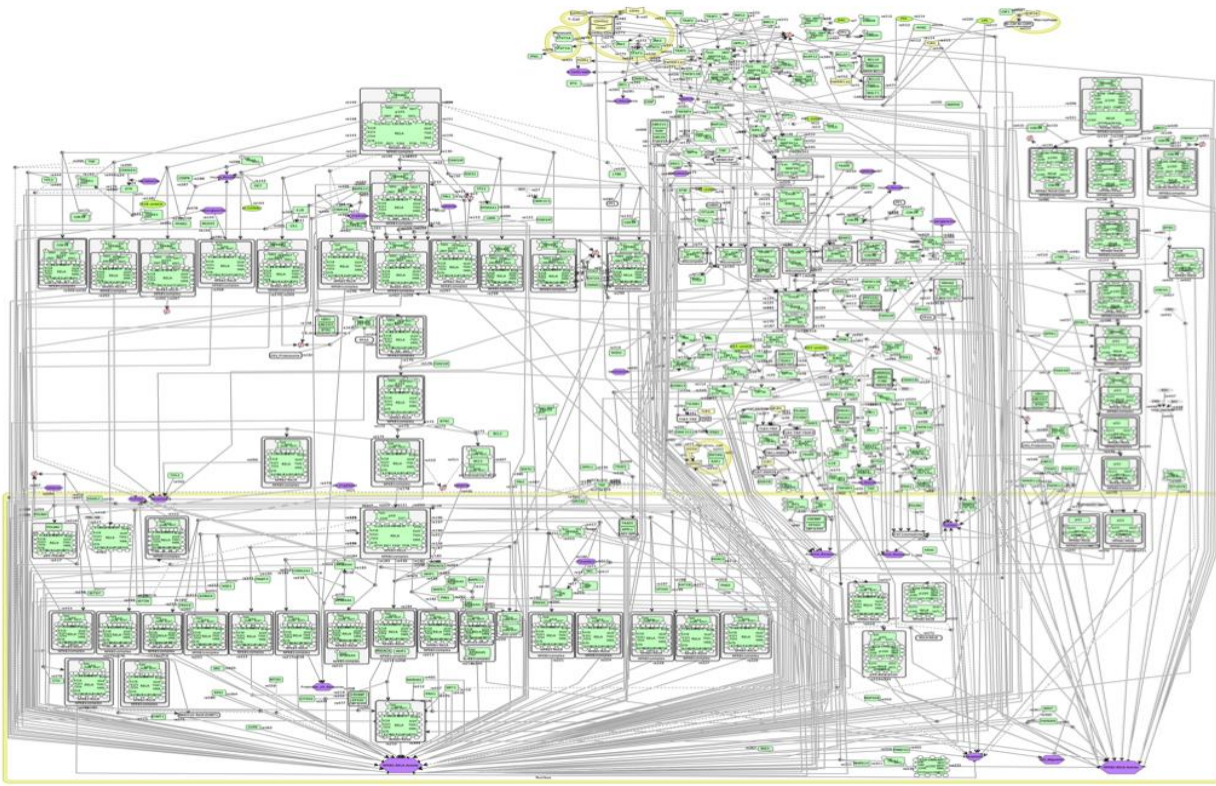
**b.** An example of positive influence reaction in the model: UBE2I sumoylate p100 in NFKB2:RELB complex at Lys689.

**c.** An example of negative influence reaction in the model: The dephosphorylation of IKKcomplex (IKKBK) and hence its inhibition requires both protein phosphatase 1 complex and adaptor protein CUEDC2.

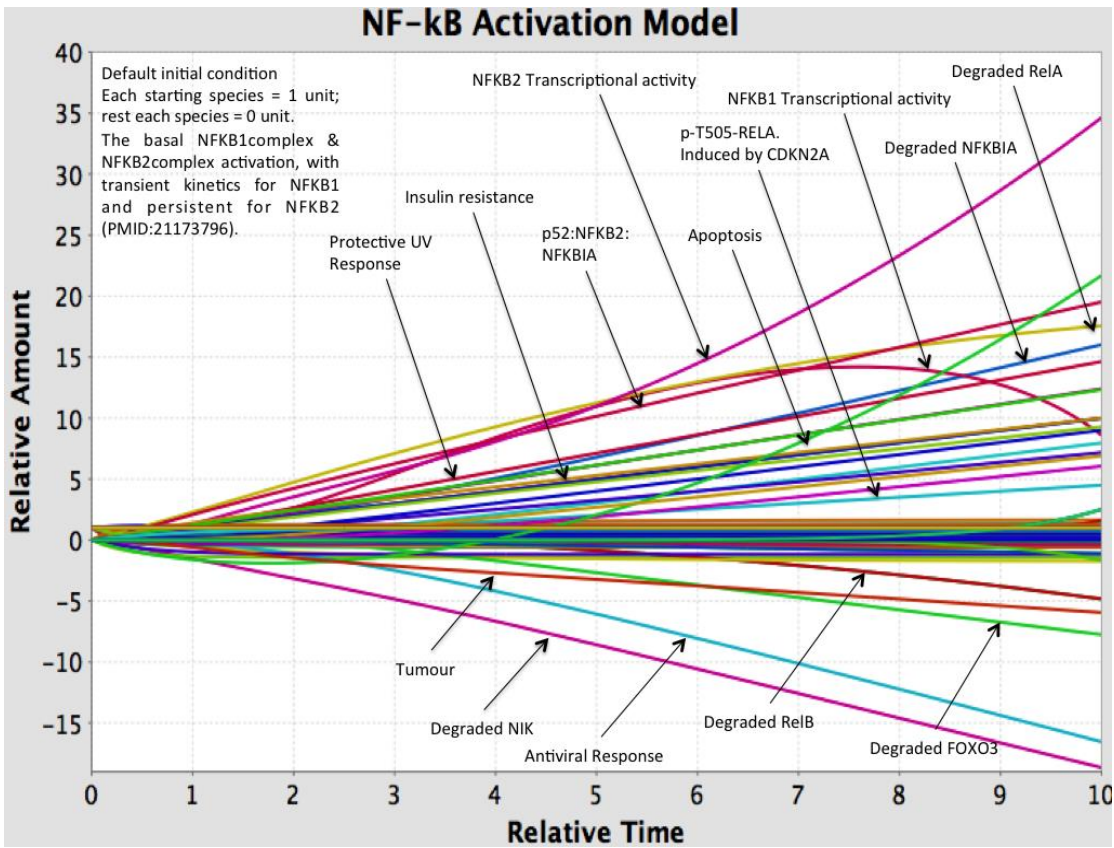
**d.** An example of the dissociation and degradation reaction in the model: The complex containing phosphorylated NFKBIA, p105, and RelA is dissociated with simultaneous ubiquitination of phosphorylated NFKBIA by IκB degradation complex (UBA1:UBE2D3:BTRC). Dissociated Ub-K21,K22-p-S32,S36-NFKBIA is subsequently degraded by 26S proteasome complex.

**e.** An example of agonist-antagonist reaction in the model: NSD1 positively influences the methylation of RelA at

K218, K221 whereas KDM2A positively influences the demethylation of RelA at K218, K221 residues.

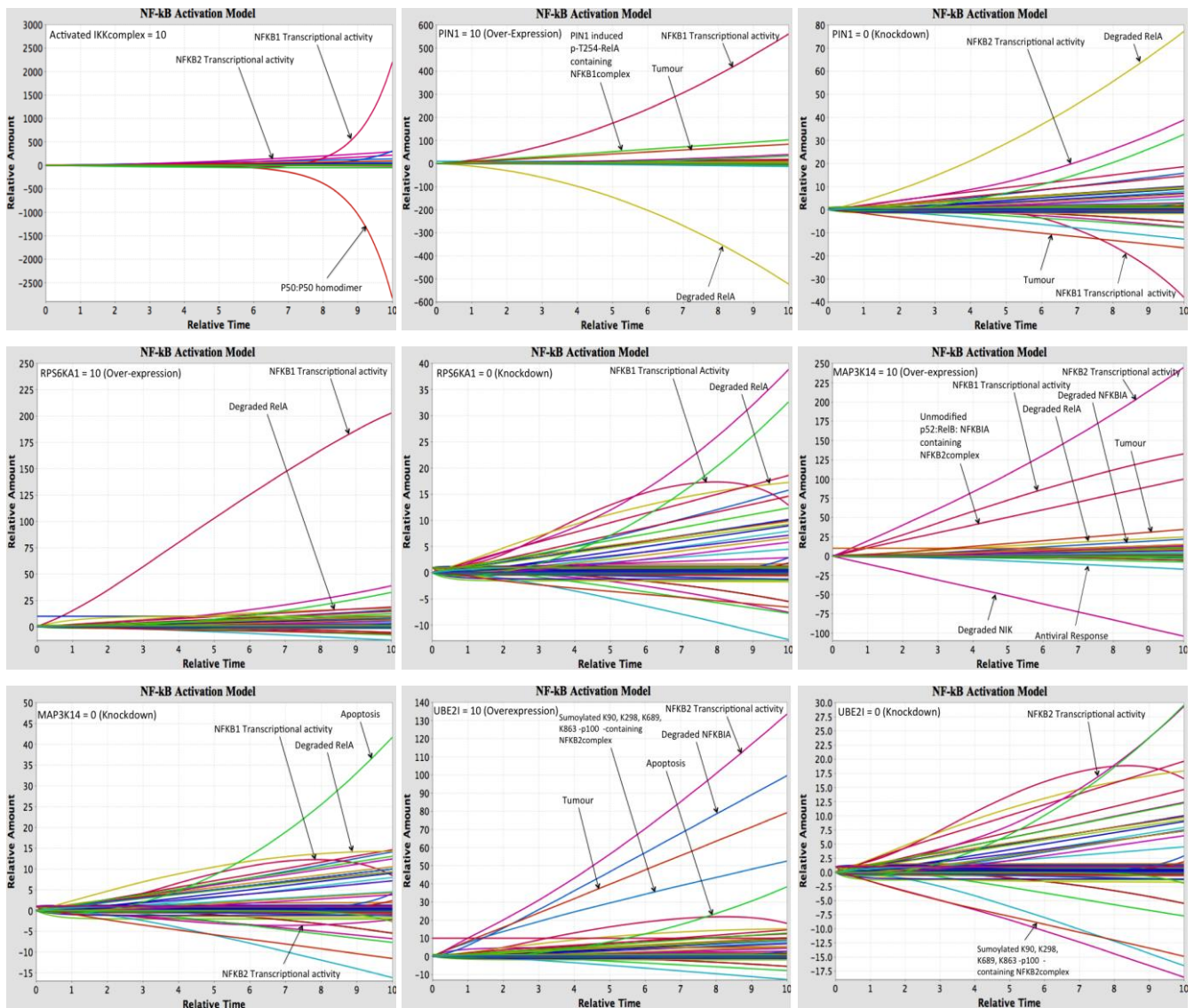


**Figure 2:** Snapshot for NF- $\kappa$ B pathway network Model including all the post-translational modifications reported for both canonical and non-canonical pathways in CellDesigner.



**Figure 3:** Output of simulation at default condition. The transient nature of activation of NFKB1 is coupled by gradual increase in Degraded RelA. The persistent nature for NFKB2 activity coupled with decrease in Degraded RelB is also observed.





**Figure 4:** NF- $\kappa$ B activation.

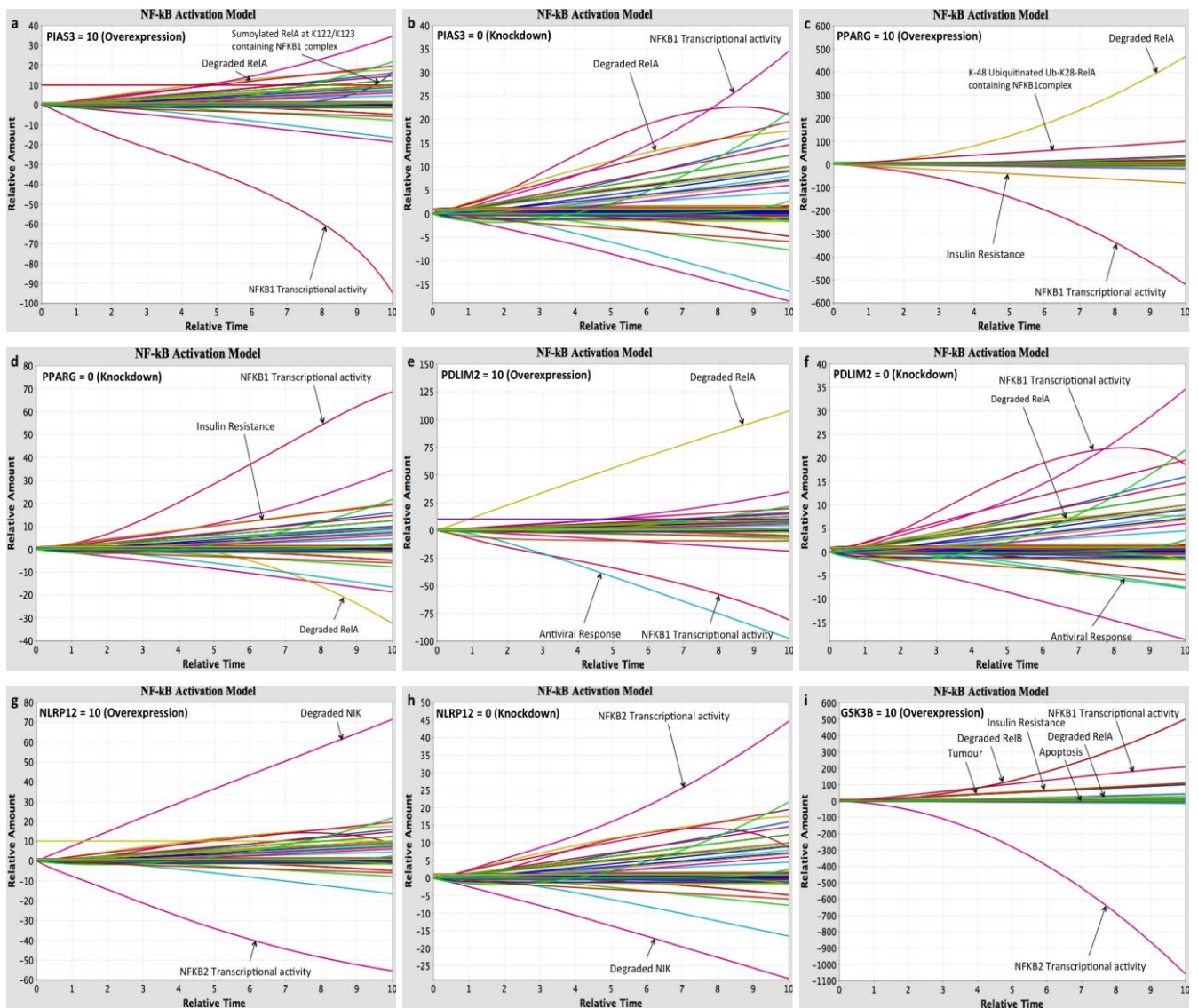
(a) Activated IKKcomplex. IKKcomplex is activated through upstream signals. Upon activation, NFKBIA is phosphorylated and degraded, and inhibits p50 homodimer binding to DNA thereby activating NFKB1:RELA complex.

(b) PIN1 overexpression and (c) PIN1 knockdown. PIN1 induces T254-RelA phosphorylation and stabilizes it, while SOCS1 induces RELA degradation in the absence of p-T245-RELA. Thus, inhibition of RELA degradation by PIN1 overexpression activates NFKB1:RELA complex;

(d) RPS6KA1 overexpression and (e) RPS6KA1 knockdown. RPS6KA1 catalyzes S536-RELA phosphorylation thereby activating it. This event, which is induced by TP53, does not require NFKBIA phosphorylation and degradation, and therefore RPS6KA1 overexpression increases NFKB1:RELA complex activity, though that is not sufficient for constitutive activation. RPS6KA1 knockdown has very little effect on NFKB1:RELA activity due to redundant activity of other modifiers.

(f) MAP3K14 overexpression and (g) MAP3K14 knockdown. MAP3K14 catalyzes the S866, S870-p100 phosphorylation thereby priming p100 (NFKB2) for proteolytic processing and activating NFKB2:RELB activity. Also, like many modifiers that phosphorylate S536-RELA, MAP3K14 phosphorylates and activates NFKB1:RELA activity. Thus, while the overexpression of MAP3K14 increases both NFKB1:RELA and NFKB2:RELB activity, its knockdown only reduces the NFKB2:RELB activity and has negligible effect on NFKB1:RELA activity.;

(h) UBE2I overexpression and (i) UBE2I knockdown. UBE2I induces p100 basal SUMO modification and thereby activates NFKB2:RELB complex. So, while it has no effect on NFKB1:RELA activity, the UBE2I overexpression increases NFKB2:RELB activity and its knockdown decreases NFKB2:RELB activity.



**Figure 5:** NF- $\kappa$ B activity inhibition.

a,b: PIAS3 overexpression and knockdown. PIAS3 mediates RELA sumoylation at K121/K122, thereby inhibiting it. PIAS3 exhibit its effect once RELA is bound to DNA in the nucleus, so on overexpression, there is late increase in sumoylated-RELA specie that is negatively associated with decrease in the NFKB1:RELA activity. The knockdown of PIAS3 though increases NFKB1:RELA activity moderately.

c,d: PPARG overexpression and knockdown. PPARG catalyzes the K48 linked-polyubiquitin delivery to K28-RELA leading to RELA ubiquitination and degradation, and subsequent reduction in NFKB1:RELA activity. Since PPARG exhibits this activity outside the nucleus, the both overexpression and knockdown of PPARG decreases and increases the NFKB1:RELA activity respectively.

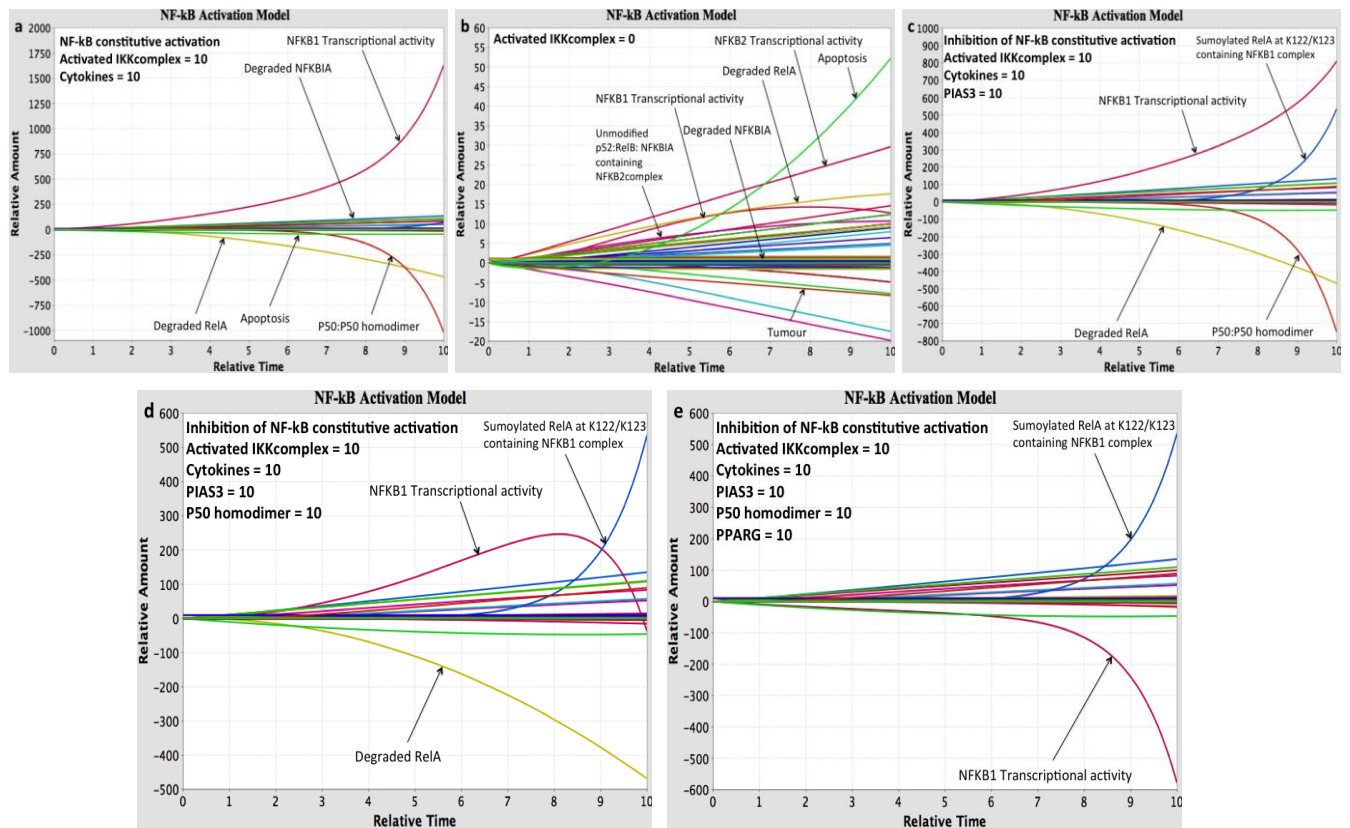
e,f: PDLIM2 overexpression and knockdown. PDLIM2 induces the RELA polyubiquitination triggering RELA degradation and thereby reduces NFKB1:RELA activity. On overexpressing PDLIM2, the RELA degradation increases followed by decrease in NFKB1:RELA activity, whereas knockdown of PDLIM2 slightly increases the NFKB1:RELA activity with accompanied slight decrease in Degraded RELA.

g,h: NLRP12 overexpression and knockdown. NLRP12 induces the proteasome-mediated MAP3K14 degradation thereby reducing the NFKB2:RELB activity. The NLRP12 overexpression thus increases the MAP3K14 (NIK) degradation followed by decrease in NFKB2:RELB activity. On the contrary, the knockdown of NLRP12 exhibit the increase in NFKB2:RELB activity and decrease in degraded MAP3K14.;

(i) GSK3B overexpression. GSK3B is reported to catalyze p100 and RELB phosphorylation, thereby mediating their degradation and reducing NFKB2:RELB activity. Effect of GSK3B on NFKB1:RELA activity is of mixed importance since though it mediates RELA degradation it also activates I $\kappa$ BKG and p105 (NFKB1) through phosphorylation. Thus, the overexpression of GSK3B is accompanied with increase in RELB degradation and consequent acute decrease in the



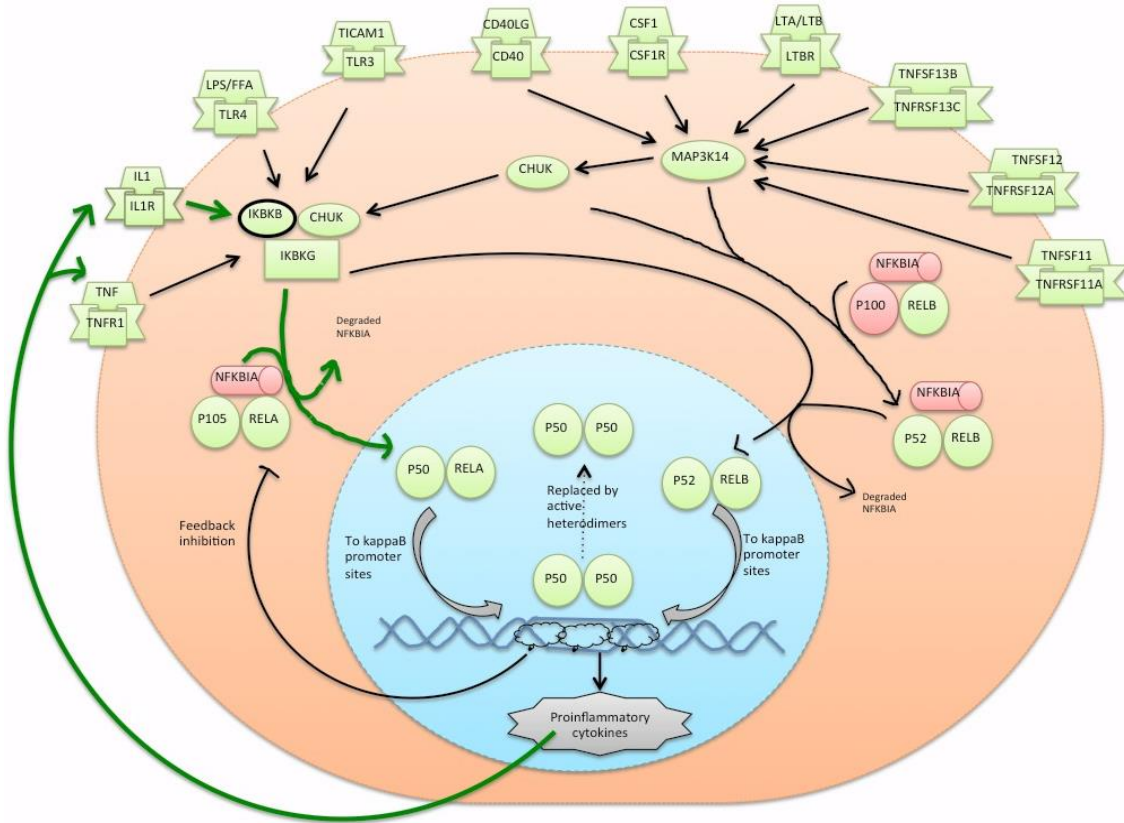
NFKB2:RELB activity. Interestingly NFKB1:RELA activity increases, exhibiting more uniform increasing trend than transient trend as observed in the default simulation run. GSK3B knockdown does not reveal any major change (figure not shown).



**Figure 6:** NF- $\kappa$ B constitutive activation and its inhibition through pathway simulations.

- (a) IKKcomplex is reported for its prominent role in NF- $\kappa$ B activation, which is achieved through phosphorylation mediated NFKBIA degradation, S536-RELA phosphorylation and activation, and, remove the inhibition by p50homodimer at  $\kappa$ B promoter sites. The NF- $\kappa$ B induces the expression of various cytokines that bind to their respective receptors and activate NF- $\kappa$ B via TRAF2/3/6-TAK1-IKKcomplex, which forms a vicious circle of activation. Thus, overexpressing both IKKcomple and cytokine, constitutively activate the NF- $\kappa$ B.;
- (b) Knockdown of IKKcomplex. In the context of normal NF- $\kappa$ B signaling, the NF- $\kappa$ B shows activity even in the absence of IKKcomplex. Therefore it mandates the intervention of NF- $\kappa$ B subunits for its activity suppression.;
- (c) Inhibition of NF- $\kappa$ B constitutive activity by PIAS3 overexpression. PIAS3 overexpression fetched down the NF- $\kappa$ B activity but was not sufficient for complete inhibition.;
- (d) Inhibition of NF- $\kappa$ B constitutive activity by PIAS3 and p50 homodimer overexpression. P50homodimer at basal condition bind to the  $\kappa$ B promoter sites and inhibit NFKB1:RELA activity. The activating stimuli are shown to expel this inhibition. The overexpression of p50 homodimer compete with binding of NFKB1:RELA to  $\kappa$ B sites, and so reduce the NFKB1:RELA activity, though that is still not adequate.;
- (e) Complete inhibition of NF- $\kappa$ B constitutive activity by PIAS3, p50 homodimer and PPARG overexpression. PPARG mediates RELA degradation through ubiquitination. The PPARG induced RELA degradation, complemented by p50homodimer mediated NF- $\kappa$ B DNA binding inhibition and PIAS3 mediated NF- $\kappa$ B activity inhibition, reverses the NF- $\kappa$ B constitutive activation.

a



b

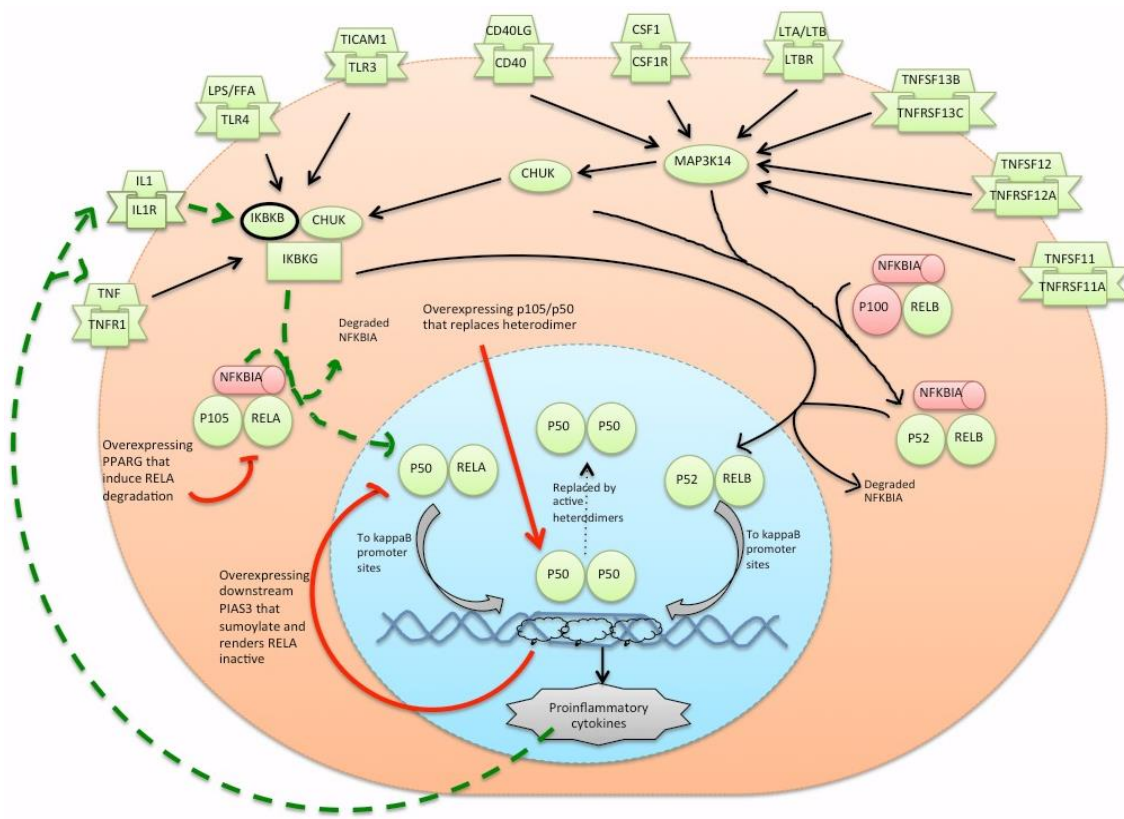


Figure 7: Schematic for constitutive NF-κB complex activation and inhibition.

(a) Schematic for Activation of NF- $\kappa$ B complex. IKKcomplex is activated by multiple ligand-receptor pair that on activation mediates the p50:RELA complex nuclear translocation which then induces downstream gene expression (PMID:17183360, 15122352, 18927578). IKKcomplex (IKBKB) is primarily active during the NF- $\kappa$ B constitutive activation (PMID: 9399940, 9199297, 10090947, 11801732, 20224721, 28215229) via expression of pro-inflammatory cytokines and is self-propagating (PMID: 25652642, 29037210). The activated NF- $\kappa$ B induces the expression of genes which mediates its feedback inhibition (PMID: 19233657) but is insufficient due to the ubiquitous expression of NF- $\kappa$ B subunits. The bold green arrow denotes the flow for NF- $\kappa$ B constitutive activation.

(b) Schematic for reversing the constitutive activation of NF- $\kappa$ B complex. The inhibition of NF- $\kappa$ B constitutive activation is achieved by integrating multiple strategies. Overexpressing PIAS3 that induces RELA - K37, K122 SUMOylation where DNA bound RelA is the preferred target (PMID: 22649547); overexpressing the p50 that represses pro-inflammatory cytokine expression (PMID: 1604322); and overexpression of PPARG that induces RELA degradation via K48-linked ubiquitination at RELA-K28. The bold red arrow denotes the schematic for the inhibition of NF- $\kappa$ B constitutive activation.

## Tables:

Table 1: Post-translational Modifications of NF- $\kappa$ B subunits included in the NF- $\kappa$ B signaling Network Model

| S. No. | Post-translational modifications | Amino acid Residue modified | Post-translational modifying enzymes/modifiers                                    | Effect on NF- $\kappa$ B complex activity | Reference (PMID)   |
|--------|----------------------------------|-----------------------------|---|---|--|
| 1      | Phosphorylation of RelA          | T254                        | Unknown kinase induced by PIN1  | NFKB1 activity increases                  | 26999213, 14690596   |
| 2      | Phosphorylation of RelA          | S276                        | PRKACA, CSNK2B, PIM1, pS324-RPS6KA4, pS360-pT581-RPS6KA5                          | NFKB1 activity increases                  | 26999213, 9660950, 14580335, 19911008, 19933278, 12628924            |
| 3      | Phosphorylation of RelA          | S311                        | PRKCZ induced by TNF  | NFKB1 activity increases                  | 12881425   |
| 4      | Phosphorylation of RelA          | S316                        | CK1 (CSNK1D) induced by IL1B  | NFKB1 activity increases                  | 26082493   |
| 5      | Phosphorylation of RelA          | S468                        | GSK3B   | NFKB1 activity decreases                  | 15465828   |
| 6      | Phosphorylation of RelA          | T505                        | pS317-pS345-CHEK1 induced by ARF and ATR  | NFKB1 activity decreases                  | 15775976, 11390642   |
| 7      | Phosphorylation of RelA          | S529                        | CK1, CSNK2B, CSNK2A1  | NFKB1 activity increases                  | 26082493, 14580335, 9789032, 19933278                                |
| 8      | Phosphorylation of RelA          | S536                        | CK1, USP6, pS177-pS181-IKBKB, pT559-MAP3K14, pS172-TBK1, Sumo-K231-IKBKE, RPS6KA1 | NFKB1 activity increases                  | 26082493, 15489227, 22081069, 18439422, 15489227, 20188669, 15489227 |
| 9      | Phosphorylation of RelA          | Y (Unknown)                 | pS525-pS526-SYK   | NFKB1 activity increases                  | 18362147, 17012367   |
| 10     | Methylation of RelA              | R30                         | PRMT5   | NFKB1 activity increases                  | 23904475   |

|    |                         |                  |                                      |                          |                                       |
|----|-------------------------|------------------|--------------------------------------|--------------------------|---------------------------------------|
| 11 | Methylation of RelA     | K218, K221       | NSD1                                 | NFKB1 activity increases | 20080798                              |
| 12 | Methylation of RelA     | K310             | SETD6                                | NFKB1 activity decreases | 21131967, 21515635, 29435148          |
| 13 | Methylation of RelA     | K314             | SETD7                                | NFKB1 activity decreases | 19262565                              |
| 14 | Acetylation of RelA     | K122, K123       | KAT2B, EP300                         | NFKB1 activity decreases | 12419806                              |
| 15 | Acetylation of RelA     | K310             | CREBBP: EP300                        | NFKB1 activity increases | 16135789                              |
| 16 | Ubiquitination of RelA  | K28              | PPARG                                | NFKB1 activity decreases | 23250430                              |
| 17 | Ubiquitination of RelA  | Ub (Unknown)     | PDLIM2                               | NFKB1 activity decreases | 17468759                              |
| 18 | Sumoylation of RelA     | K122, K123       | PIAS3                                | NFKB1 activity decreases | 22649547                              |
| 19 | O-GlcNAcylation of RelA | T352             | OGT induced by hyperglycemia         | NFKB1 activity increases | 18988733                              |
| 20 | Sulphydration of RelA   | C38              | CTH                                  | NFKB1 activity increases | 22244329                              |
| 21 | Nitrosylation of RelA   | C38              | NOS2                                 | NFKB1 activity decreases | 8710491, 12091382, 17720813           |
| 23 | Phosphorylation of p50  | S20              | PRKDC                                | NFKB1 activity increases | 20966071                              |
| 23 | Phosphorylation of p50  | S328             | ATR, pS317-pS345-CHEK1               | NFKB1 activity decreases | 25590437, 26291483                    |
| 25 | Phosphorylation of p50  | S337             | PRKACA                               | NFKB1 activity decreases | 12947093, 15642694                    |
| 26 | Phosphorylation of RelB | S472             | pS177-pS181-IKBKB, GSK3A, pS176-CHUK | NFKB2 activity increases | 8657102, 20622870, 23547054, 12709443 |
| 27 | Phosphorylation of RelB | S552             | GSK3B                                | NFKB2 activity decreases | 21217772, 11781828                    |
| 28 | Sumoylation of p100     | K298, K689, K863 | UBE2I                                | NFKB2 activity increases | 18617892                              |

Note: The proteolytic processing of p105 to p50 and p100 to p52, degradation of NFKBIA, and post-translational modifications of post-translational modifying enzymes (IKKcomplex, TAK1, TBK1, etc.), which are integrated in model are not included in this list.

Table 2: Perturbations and simulation runs of 10 cases included in NF- $\kappa$ B model

|   | Perturbation   | Experimental Evidence   | Experiment specification  | Variable setting | Model Outcome (Supplementary File2)  |
|---|----------------|---|---|------------------|--|
| 1 | PIN1 Knock out | Pin1 deficient mice and cells are refractory to NF-kappaB activation (Immuno staining). Pin1 does not modulate the IKK Activity and Phosphorylation status of I $\kappa$ B $\alpha$ but restricts the | Cell line: breast cancer array, HeLa cells, Pin1 <sup>-/-</sup> or WT Mouse embryonic fibroblasts | PIN1 = 0 unit    | RelA degradation increases and active state of NF $\kappa$ B1 complex decreases. |



|   |                         |  |  |  |   |
|---|-------------------------|--|--|--|---|
|   |                         | interaction between p65 and I $\kappa$ B $\alpha$ (GST pull down and immunoblot) [PMID: 14690596]  |  |  |   |
| 2 | PIN1 overexpression     | Twenty out of twenty-five cancer samples that contained high Pin1 had a strong nuclear accumulation of p65 (immunoblot assay) and phosphorylated RelA at T254. Overexpression of Pin1 activated, whereas depletion of Pin1 suppressed, -NF- $\kappa$ B activity in a dose-dependent manner (Luciferase assay) [PMID: 14690596] | Cell line: breast cancer array, HeLa cells, Pin1 <sup>-/-</sup> or WT Mouse embryonic fibroblasts  | PIN1 = 10 units                            | The activity of NF $\kappa$ B1 complex increases, while RelA degradation decreases.   |
| 3 | PRKCZ overexpression    | Phosphorylation of RelA at Ser311 by PRKCZ (assayed through ITC and immunoblot assay) blocked the binding of EHMT1 to me-K310-RelA (pulldown assay) and relieved repression of the target genes. [PMID: 12881425; 21131967]  | Cell line: HEK 293T cells; wt and PRKCZ <sup>-/-</sup> mouse embryonic fibroblasts; RelA mutant through site-directed mutagenesis (PMID: 21131967) | PRKCZ = 10 units, SETD6 = 1, default value | The increased levels of PRKCZ, reverses the RelA inhibition by SETD6, thereby elevating the active state of NF $\kappa$ B1 complex. |
| 4 | NKIRAS1 overexpression  | $\kappa$ B-Ras suppressed phosphorylation at serine 276 on the p65/RelA subunit (GST Pulldown), resulting in decreased interaction between p65/RelA and the transcriptional coactivator p300 (immunoprecipitation), thereby reducing NF- $\kappa$ B activity (Luciferase assay). [PMID: 20639196]                              | Cell line: HEK293T   | NKIRAS1 = 10 units                         | There is a decrease in the active state of NF $\kappa$ B1 complex.  |
| 5 | NSD1 overexpression     | NSD1 overexpression mediates the K218, K221-RelA methylation (mass spectrometry) increasing the NF-kappaB activity (luciferase assay). [PMID: 20080798]  | Cell line: 293C6, HT29, and mutant Z3 cells; wt-RELA and RELA <sup>-/-</sup> MEF   | NSD1 = 10 units                            | Increases the active state of NF $\kappa$ B1 complex.   |
| 6 | KDM2A overexpression    | Overexpression of KDM2A mediates K218, K221-RelA demethylation inhibiting NF-kappaB activity (luciferase assay). [PMID: 20080798]  | Cell line: 293C6, HT29, and mutant Z3 cells; wt-RELA and RELA <sup>-/-</sup> MEF   | KDM2A = 10 units                           | Leads to decrease in active state of NF $\kappa$ B1 complex.  |
| 7 | Overexpression of PIAS4 | Overexpression of PIAS4 (siRNA and immunoblotting) enhances  | Cell line: HEK293 cells  | PIAS4 = 10 units                           | Enhances the active state of NF $\kappa$ B1 complex.  |

|    |                             |   |  |   |  |
|----|-----------------------------|---|--|---|--|
|    |                             | NEMO sumoylation (immunoblotting) and NFKB1 complex activation (Electrophoretic mobility shift assay) [PMID: 16906147]  |  |   |  |
| 8  | Overexpression of SENP2     | SENP2 can efficiently associate with NEMO deSUMOylating it (immunoblotting and EMSA), and thereby inhibit NFKB1 transcriptional activation (luciferase assay) [PMID: 21777808; 16862178]  | Cell line: HEK293 cells; Wt, Senp1 <sup>-/-</sup> and Senp2 <sup>-/-</sup> MEF | SENP2 = 10 units  | Led to acute reduction in NFKB1 complex activation. Also it counteracts the effect of increased levels of PIAS4.   |
| 9  | Activation of MAP3K7 (TAK1) | TAK1 is activated by various cytokines (immunoprecipitation and immunoblot), including TNF, IL1, IL6, IL1B, which by activation of IKKB, increases transcriptional activation of NFKB1 complex (RT PCR and ELISA) [PMID: 20038579; 26491199; 25028512]. | Human Neutrophils suspension culture.  | Cytokine = 10 units (a specie describing IL1, IL1B, IL6 conglomerate) | The level of TAK1 is increased, which consequently led to increase in active state of NKB1 complex.                |
| 10 | Overexpression of CDKN2A    | By inducing T505-RelA phosphorylation through ATR and CHEK1 (immunoblot), CDKN2A mediates repression of NFKB1 complex transcriptional activity (luciferase assay), and promotes cell death (Trypan Blue Viability Assay)[PMID: 15775976; 11390642].     | Cell line: NARF2 and NARF2-E6 and Hs68 E2F1-ER                                 | CDKN2A = 10 units   | The level of p-T505-RelA increases, subsequently promoting cell death and reduction in NFKB1 complex active state. |