

## Supplementary Mathematical Methods

The mathematical analysis of the NF- $\kappa$ B pathway regulation is discussed in two sections. First, we consider TNF $\alpha$ -TNFR1 interactions in the microfluidic chamber. Next, in a separate single-cell stochastic model, we analyze dynamics of NF- $\kappa$ B controlled by interlinked negative feedback loops mediated by A20 and I $\kappa$ B $\alpha$ .

### 1. TNF $\alpha$ -TNFR1 kinetics

The kinetics of TNF $\alpha$  molecules in the experimental chamber involves four processes:

- 1) TNF $\alpha$  degradation
- 2) TNF $\alpha$ -TNFR1 association
- 3) TNF $\alpha$ -TNFR1 dissociation
- 4) Internalization of TNFR1

In addition to binding dissociation, diffusion and internalization rates, these processes are further controlled by the two parameters specific for the experimental apparatus used and conditions tested: (1) chamber volume per cell and (2) the initial TNF $\alpha$  concentration. The chamber volume - which is often omitted when calculating receptor dynamics - is of key importance especially at low volumes since it determines the available number of TNF $\alpha$  (trimers) molecules per cell. It is especially important at low doses when this number is smaller than number of TNF $\alpha$  receptors available for binding (thousands per cell). In the 35 nanoliter microfluidic chambers we used, the effective loss of free TNF $\alpha$  is due to natural degradation (half life~1h) and to binding to TNF $\alpha$  receptors, which are then internalized. According to Grell et al. (1998), the TNF $\alpha$ -TNFR1 dissociation half-time (33 min) is about twice longer than the internalization half-time (10-20 min), as a result bound TNF $\alpha$  molecules are mostly internalized than dissociate. At low TNF $\alpha$  doses the internalization mechanism becomes more effective than natural degradation and the effective loss of free TNF $\alpha$  in our experimental conditions is much faster than degradation (Fig. M1). The effective free TNF $\alpha$  loss rates in our microfluidic chambers are estimated as  $2 \times 10^{-4} \text{ s}^{-1}$ ,  $7 \times 10^{-4} \text{ s}^{-1}$ ,  $7.7 \times 10^{-4} \text{ s}^{-1}$  and  $8.3 \times 10^{-4} \text{ s}^{-1}$  for 10ng/ml, 1ng/ml, 0.1ng/ml, and 0.01ng/ml TNF $\alpha$  concentrations, respectively. These rates were used in main model for simulations of cell activation and response dynamics in the microfluidic chamber.

For experimental chambers with larger volume, the peak numbers of bound TNF $\alpha$ -TNFR1 ligand-receptor complexes are similar to those in lower volumes (Figure M.2). However, at larger volumes significantly higher level of bound complexes are maintained after the peak, which should lead to prolonged activity of TNF $\alpha$  induced NF- $\kappa$ B (i.e. larger fraction of active cells, late activating cells) in such volumes. The parameters used in these calculations can be seen in Table 1.

**Table 1.** Parameters for TNF $\alpha$ -TNFR1 kinetics.

symbol	value	Reaction and reference
$k_{on}$	$1.83 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$	$\text{TNF} + \text{TNFR1} \rightarrow \text{TNF TNFR1}$ Grell et al. (1998)
$k_{off}$	$3.48 \times 10^{-4} \text{ s}^{-1}$	$\text{TNF TNFR1} \rightarrow \text{TNF} + \text{TNFR1}$ Grell et al. (1998)
$k_{int}$	$7.70 \times 10^{-4} \text{ s}^{-1}$	$\text{TNF TNFR1} \rightarrow \text{TNF TNFR1}_{int}$ Grell et al. (1998)
$c_{deg}$	$2 \times 10^{-4} \text{ s}^{-1}$	$\text{TNF} \rightarrow \emptyset$ this study

The equations describing receptor dynamics are:

*Free TNF $\alpha$*

$$\frac{d}{dt} \text{TNF}(t) = -k_{on} \times \text{TNF}(t) \times \text{TNFR1}(t) - c_{deg} \times \text{TNF}(t) + k_{off} \times (\text{TNF} | \text{TNFR1})(t)$$

*Free TNFR1 receptors*

$$\frac{d}{dt} \text{TNFR1}(t) = -k_{on} \times \text{TNF}(t) \times \text{TNFR1}(t) + (c_{deg} + k_{off}) \times (\text{TNF} | \text{TNFR1})(t)$$

*TNFR1-TNF $\alpha$  complexes*

$$\frac{d}{dt} (\text{TNF} | \text{TNFR1})(t) = k_{on} \times \text{TNF}(t) \times \text{TNFR1}(t) - (c_{deg} + k_{off} + k_{int}) \times (\text{TNF} | \text{TNFR1})(t)$$

*TNFR1-TNF $\alpha$  complexes internalized*

$$\frac{d}{dt}(\text{TNF} | \text{TNFR1})_{\text{int}}(t) = k_{\text{int}} \times (\text{TNF} | \text{TNFR1})(t)$$

Binding of the TNF $\alpha$  to TNFR1 does not necessarily lead to formation of the active TNF $\alpha$  receptor complexes that lead to NF- $\kappa$ B pathway activation. First, the downstream signaling requires TNFR1 trimerization, which may take place before TNF $\alpha$  trimer binding (see Chan 2007 for discussion). Next, the adapter protein TRADD binds and serves as an assembly platform for TRAF2 and RIP. At this step the IKKK (IKK kinase) maybe activated, alternatively TRADD and RIP associate with FADD and cell-death-initiation caspase 8 (complex II), which then activates executioner caspase 3 leading to apoptic DNA fragmentation. As a result one can expect that the number of complexes that serve as IKKK activation platform is smaller than the number of bound TNF $\alpha$  trimer molecules.

## 2. NF- $\kappa$ B pathway regulation in the single cell

The current model follows our previous model (Lipniacki et al. 2007) that involved two-compartment kinetics of NF- $\kappa$ B and its inhibitors A20 and I $\kappa$ B $\alpha$ , and allowed for analysis of single cell responses to TNF $\alpha$  stimulation of arbitrary and time dependent intensity, in both wild type and A20 deficient cells. The model combines the two feedback NF- $\kappa$ B-I $\kappa$ B $\alpha$ -A20 regulatory module with the signal transduction-amplification cascade, which transmits the signal from the TNFR1 receptors.

### Receptor activation and signal transduction pathway

The single event of receptor activation is amplified by the three-step signal transduction cascade involving activation of kinases named IKKK (IKK activating kinase) and IKK. Biologically, there are at least two kinases involved in this process: MEKK3 and TAK1. We assume that IKKK migrates toward the receptor and is activated at the receptor (transformation from IKKK<sub>n</sub> to IKKK<sub>a</sub>). Active IKKK<sub>a</sub> molecules activate IKK molecules (transformation from IKK<sub>n</sub> to IKK<sub>a</sub>). Since full activation of IKK requires its phosphorylation at two serine residues Ser177 and Ser181 (Delhase et al., 1999), we assume in this model that IKK activation rate is proportional to square of active IKKK<sub>a</sub>. In turn active IKK<sub>a</sub> phosphorylate I $\kappa$ B $\alpha$  molecules leading to their

ubiquitination and degradation. It is assumed that the total number of IKKK and IKK molecules (as well as of the NF- $\kappa$ B molecules) is constant; i.e., their degradation is balanced by production, but both terms are omitted in the mathematical representation. The IKKK may exist in one of two states: native neutral IKKK<sub>n</sub>, specific to unstimulated resting cells; and active IKKK<sub>a</sub>. IKK complexes, consisting of catalytic subunits IKK $\alpha$  and IKK $\beta$  and regulatory subunits IKK $\gamma$ , may exist in one of four states: native neutral (denoted by IKK<sub>n</sub>), specific to unstimulated resting cells; active (denoted by IKK<sub>a</sub>) arising from IKK<sub>n</sub> via phosphorylation of serines 177 and 181 of IKK subunits (Delhase et al., 1999) upon the IKKK<sub>a</sub> induced activation; inactive (denoted by IKK<sub>i</sub>) but different from the native neutral form, arising from IKK<sub>a</sub> possibly due to overphosphorylation; and transient between IKK<sub>i</sub> and IKK<sub>n</sub>, also inactive, denoted by IKK<sub>ii</sub>.

### The NF- $\kappa$ B-I $\kappa$ B $\alpha$ -A20 regulatory module

In resting cells, the unphosphorylated I $\kappa$ B $\alpha$  binds to NF- $\kappa$ B and sequesters it in an inactive form in the cytoplasm. IKK<sub>a</sub> mediated phosphorylation of I $\kappa$ B $\alpha$  leads to its degradation and releases the main activator NF- $\kappa$ B, which then enters the nucleus and triggers transcription of the two inhibitors and numerous other genes. The newly synthesized I $\kappa$ B $\alpha$  leads NF- $\kappa$ B out of the nucleus and sequesters it in the cytoplasm.

IKK inactivation is controlled by the second inhibitor A20, which is strongly up-regulated by NF- $\kappa$ B (Krikos et al., 1992). The exact mechanism of A20 action is still not fully resolved. Here, we assume that A20 acts in two ways: (1) It initiates degradation of RIP, the key component of the TNFR1 receptor complex (Wertz et al., 2004), which attenuates the activity of receptors, and (2) it directly associates with IKK (Zhang et al., 2000) enhancing IKK<sub>a</sub> conversion to catalytically inactive form IKK<sub>i</sub> (the conversion from IKK<sub>a</sub> to IKK<sub>i</sub> takes place also in A20 deficient cells but at a slower rate). The exact mechanism of IKK inactivation also remains unresolved: According to Delhase et al., (1999) IKK inactivates via autophosphorylation of serines in IKK C-terminal region. However, Schomer-Miller et al. (2006) found that this autophosphorylation does not diminish IKK activity and suggested that phosphorylation of serines 740 and 750 in NBD/BD domain of IKK may have a regulatory role and that their phosphorylation may downregulate IKK activity. The form IKK<sub>i</sub> spontaneously converts into IKK<sub>n</sub> through several inactive intermediate forms collectively denoted by IKK<sub>ii</sub>. The number of these forms may be large since there are at least 16 serine residues in IKK, which may be involved in regulation of IKK activity according to Schomer-Miller et al. (2006). This intermediate step is introduced in our model to account for the

delay needed to process the inactivated form IKKi into native state IKKn. This effect is manifested in A20<sup>-/-</sup> cells under persistent or long lasting TNF $\alpha$  stimulation as a down-regulation of IKK activity at about 30 minutes followed by a higher plateau. According to our model in the first minutes of high dose TNF $\alpha$  stimulation most of IKKn is used up so the IKK activation rate is low. The activation rate and the level of IKKa may increase only after some IKKn is recovered via the intermediate form IKKii.

Recently, White and colleagues proposed that A20 does not act between IKKa and IKKi, but slows down the restoration of IKKn from inactive form IKKi (Ashall et al. 2009). Such assumption allows for reproduction of the system responses to pulse-pulse-pulse stimulation in a very broad range (4 orders of magnitude) of A20 degradation parameters. However, after such a modification, we encounter difficulties in reproducing the kinetics of A20<sup>-/-</sup> cells. Our current model, after adjusting the parameters, properly reproduces short pulse experiments by Ashall et al. (2009).

The inhibitor I $\kappa$ B $\alpha$  migrates between the nucleus and cytoplasm and forms complexes with NF- $\kappa$ B molecules. The nuclear I $\kappa$ B $\alpha$ -NF- $\kappa$ B complexes quickly migrate into the cytoplasm. The second inhibitory protein A20 is considered only in the cytoplasm where it triggers the inactivation of IKK. It is assumed that the transformation rate from IKKa into IKKi is the sum of the constant term and a term proportional to the amount of A20. The transcriptional regulation of A20 and I $\kappa$ B $\alpha$  genes is governed by the same rapid elongation regulatory mechanism with a rapid coupling between NF- $\kappa$ B binding and transcription. The mechanisms for NF- $\kappa$ B dependent regulation of I $\kappa$ B $\alpha$  and A20 are based on the control of transcriptional elongation. In this situation, stalled RNA polymerase II is rapidly activated by NF- $\kappa$ B binding to enter a functional elongation mode, and requires continued NF- $\kappa$ B binding for re-initiation. This is represented in our model by tight coupling of NF- $\kappa$ B binding to mRNA transcription. We assume that all cells are diploid, and both A20 and I $\kappa$ B $\alpha$  genes have two potentially active homologous copies, each of which is independently activated due to binding of NF- $\kappa$ B molecule to a specific regulatory site in gene promoter. Following our previous studies (Lipniacki et al. 2006 and 2007) we made the simplifying assumption that each gene copy may exist only in one of two states; active and inactive. When the copy is active the transcription is initiated at a high rate, when the copy is inactive transcription is inhibited. The gene copy becomes inactive when the NF- $\kappa$ B molecule is removed from its regulatory site due to the action of I $\kappa$ B $\alpha$  molecules, which bind to DNA-associated NF- $\kappa$ B, exporting it out of the nucleus.

## Sources of heterogeneity in NF-κB regulation

We consider two types of molecular noise sources in this model:

1. **Extrinsic noise**: due to different levels of total NF-κB and TNFR-1 across the population. Our experiments show heterogeneity in total cell fluorescence, indicating that RelA levels in individual cells are not equal. Furthermore, many cell types including 3T3's we used have considerable variation in their TNFR-1 levels, which leads to variable sensitivity to TNFα. Accordingly, we assume in our model that total NF-κB and TNFR-1 follow the log normal distributions defined as

$$f = \frac{A}{x\sigma\sqrt{2\pi}} \text{Exp}\left(-\frac{(\ln(x) - \mu)^2}{2\sigma^2}\right) \quad x > 0$$

with

$$\text{mean} = A \times \text{Exp}(\mu + \sigma^2/2), \quad \text{median} = A \times \text{Exp}(\mu), \quad \text{Var} = (\text{Exp}(\sigma^2) - 1) \text{Exp}(2\mu + \sigma^2)$$

$$\text{For NF-}\kappa\text{B we assume: } A_N = 10^5, \quad \sigma_N = 1/\sqrt{2}, \quad \mu_N = -1/4$$

$$\text{mean}_N = A_N, \quad \text{median}_N = A_N \times e^{-1/4}, \quad \text{Var}_N = A_N^2 \times (e^{1/2} - 1)$$

and for TNFR-1 receptors:

$$A_R = 2 \times 10^3, \quad \sigma_R = \sqrt{2}, \quad \mu_R = -1$$

$$\text{mean}_R = A_R, \quad \text{median}_R = A_R \times e^{-1}, \quad \text{Var}_R = A_R^2 \times (e^2 - 1)$$

2. **Intrinsic noise**: resulting from discrete regulation of receptor activity (ligand-receptor binding, trimerization and recruitment of adaptor proteins) and transcription of A20, IκBα, TNFα and reporter genes (gene- NF-κB binding), as in Lipniacki et al. (2007).

## Mathematical representation

In this work, as in our recent studies (Lipniacki et al., 2006 and 2007) we follow the method proposed by Haseltine and Rawlings (2002) and split the reaction channels into fast and slow, which results in a hybrid stochastic-deterministic model. We consider all reactions involving mRNA and protein molecules as fast and the reactions of receptor and gene activation and inactivation as slow. Fast reactions are approximated by the deterministic reaction-rate equations, whereas slow reactions are considered stochastic. In addition we consider the deterministic approximation of hybrid model consisting solely of the ordinary differential equations (ODEs). According to the above, the hybrid model consists of 16 ordinary differential equations accounting for:

- formation of the (I $\kappa$ B $\alpha$ -NF- $\kappa$ B) complexes,
- IKKK and IKK kinase activation and inactivation
- IKKa driven I $\kappa$ B $\alpha$  phosphorylation,
- A20, I $\kappa$ B $\alpha$  and phospho-I $\kappa$ B $\alpha$  protein degradation
- transport between nucleus and cytoplasm, and
- transcription and translation.

All the substrates are quantified in number of molecules. The upper-case letters denote substrates or their complexes. Nuclear amounts are represented by subscript n, while subscript c denoting amount of substrate in the cytoplasm is omitted, to simplify the notation. Amounts of the mRNA transcripts of A20 and I $\kappa$ B $\alpha$  are denoted by subscript t:

### Notation guide:

IKKn - neutral form of IKK kinase,

IKKa - active form of IKK,

IKKi - inactive form of IKK,

IKKii - inactive intermediate form of IKK,

K<sub>NN</sub> - total number of IKK=IKKn+IKKa+IKKi+IKKii molecules (assumed constant in time)

IKKKa - active form of IKKK,

IKKKn - neutral form of IKKK,

K<sub>N</sub>- total number of IKKK=IKKKn+IKKKa molecules (assumed to be constant in time)

I $\kappa$ B - cytoplasmic I $\kappa$ B $\alpha$

$I\kappa B_n$  - nuclear  $I\kappa B\alpha$ ,

$I\kappa B_t$  -  $I\kappa B\alpha$  transcript,

$I\kappa B_p$  - phosphorylated cytoplasmic  $I\kappa B\alpha$

$NF\kappa B|I\kappa B$  - cytoplasmic (NF- $\kappa$ B- $I\kappa B\alpha$ ) complexes

$NF\kappa B|I\kappa B_p$  - phosphorylated cytoplasmic  $I\kappa B\alpha$  complexed to NF- $\kappa$ B

$NF\kappa B|I\kappa B_n$  - nuclear (NF- $\kappa$ B- $I\kappa B\alpha$ ) complexes

$TNF_{ext}$  - extracellular  $TNF\alpha$  concentration [ng/ml],

$G_{I\kappa B}$  - discrete random variable, state of  $I\kappa B\alpha$  gene,

$G_{A20}$  - discrete random variable, state of A20 gene,

$G_R$  - discrete random variable, state of reporter gene,

$B$  - number of active receptors,  $M$  - total number of receptors (assumed to be constant in time)

**Table M2** Parameter set for the main model. Since all substrates are quantified in numbers of molecules the concentration does not appear in units.

symbol	value	reaction, reference
<i>The cell</i>		
$k_v = V/U$	5	ratio of cytoplasmic to nuclear volume - assumed
$M_{3T3}$	$2 \times 10^3$	Average number of TNFR1 for 3T3 cell simulations - assumed
$M_{SK-N-AS}$	$5 \times 10^3$	Average number of TNFR1 for SK-N-AS cell simulations - assumed
$M_{MEFs}$	$10^4$	Average number of TNFR1 for MEFs cell simulations - assumed
$M_{HeLa}$	$5 \times 10^2$	Average number of TNFR1 for HeLa cell simulations - assumed
$K_N$	$10^5$	number of IKKK molecules - assumed
$K_{NN}$	$2 \times 10^5$	number of IKK molecules - assumed
$NF-\kappa B_{tot}$	$10^5$	average number of NF- $\kappa$ B molecules (see text)
<i>TNFR1 activation and signal transduction cascade</i>		
$c_{deg}$	$2 \times 10^{-4} s^{-1}$	extracellular $TNF\alpha$ degradation; for simulations of cells in the microfluidic chamber these effective loss rates are used: $2 \times 10^{-4} s^{-1}$ , $7 \times 10^{-4} s^{-1}$ , $7.7 \times 10^{-4} s^{-1}$ and $8.3 \times 10^{-4} s^{-1}$ for 10ng/ml, 1ng/ml, 0.1ng/ml,



		and 0.01ng/ml respectively
$k_b$	$1.2 \times 10^{-5} \text{ s}^{-1} (\text{ng/ml})^{-1}$	receptor activation rate - fitted
$k_f$	$1.2 \times 10^{-3} \text{ s}^{-1}$	receptor inactivation rate – Grell et al., 1998
$k_a$	$2 \times 10^{-5} \text{ s}^{-1}$	IKKK activation rate - assumed
$k_i$	$10^{-2} \text{ s}^{-1}$	IKKK inactivation rate - fitted
$k_1$	$6 \times 10^{-10} \text{ s}^{-1}$	IKKn activation rate - fitted
$k_{A20}$	$10^5$	Michaelis coefficient in TNFR1 activity attenuation - fitted
$k_2$	$10^4$	Michaelis coefficient in IKKa inactivation - fitted
$k_3$	$2 \times 10^{-3} \text{ s}^{-1}$	IKKn inactivation rate - fitted
$k_4$	$10^{-3} \text{ s}^{-1}$	IKKi→IKKii and IKKii→IKKn transformation -fitted
<i>A20 and IκBα synthesis</i>		
$q_1$	$4 \times 10^{-7} \text{ s}^{-1}$	NF-κB binding at A20 and IκBα gene promoters -fitted
$q_2$	$10^{-6} \text{ s}^{-1}$	IκBα inducible NF-κB detaching from A20 and IκBα genes - fitted
$c_1$	$0.1 \text{ s}^{-1}$	inducible A20 and IκBα mRNA synthesis - assumption
$c_3$	$7.5 \times 10^{-4} \text{ s}^{-1}$	A20 and IκBα mRNA degradation
$c_4$	$0.5 \text{ s}^{-1}$	A20 and IκBα translation - fitted
$c_5$	$5 \times 10^{-4} \text{ s}^{-1}$	A20 degradation rate - fitted
<i>IκBα interactions</i>		
$a_1$	$5 \times 10^{-7} \text{ s}^{-1}$	IκBα association NF-κB - assumption
$a_2$	$10^{-7} \text{ s}^{-1}$	IκBα phosphorylation - fitted
$a_3$	$5 \times 10^{-7} \text{ s}^{-1}$	IκBα phosphorylation in IκBα/NF-κB complexes - fitted
$t_p$	$10^{-2} \text{ s}^{-1}$	degradation of phosphorylated IκBα - fitted
$c_{5a}$	$10^{-4} \text{ s}^{-1}$	spontaneous IκBα degradation - assumption
$c_{6a}$	$2 \times 10^{-5} \text{ s}^{-1}$	spontaneous IκBα degradation in IκBα/NF-κB complexes - assumption

<i>Transport</i>		
$i_1$	$10^{-2} \text{ s}^{-1}$	NF- $\kappa$ B nuclear import
$e_{2a}$	$5 \times 10^{-2} \text{ s}^{-1}$	I $\kappa$ B $\alpha$  NF- $\kappa$ B nuclear export
$i_{1a}$	$2 \times 10^{-3} \text{ s}^{-1}$	I $\kappa$ B $\alpha$ nuclear import
$e_{1a}$	$5 \times 10^{-3} \text{ s}^{-1}$	I $\kappa$ B $\alpha$ nuclear export
<i>Reporter gene parameters</i>		
$q_{1r}$	$10^{-7} \text{ s}^{-1}$	NF- $\kappa$ B binding at reporter gene promoter
$q_{2r}$	$10^{-7} \text{ s}^{-1}$	I $\kappa$ B $\alpha$ inducible NF- $\kappa$ B detaching from reporter gene
$q_{2rr}$	$10^{-3} \text{ s}^{-1}$	spontaneous NF- $\kappa$ B detaching from reporter gene
$c_{1r}$	$5 \times 10^{-2} \text{ s}^{-1}$	inducible reporter mRNA synthesis
$c_{1rr}$	$10^{-3} \text{ s}^{-1}$	reporter mRNA constitutive synthesis
$c_{3r}$	various	reporter mRNA degradation rate

## ODEs

**IKKK in active state (IKKKa):** the first term describes IKKK kinase activation, i.e. transformation from IKKKn (amount of which is  $IKKKn = K_N - IKKKa$ ) due to action of active receptors  $B(t)$ , whose activity is attenuated by  $A_{20}$ . The second term describes spontaneous inactivation of the kinase:

$$\frac{d}{dt} IKKKa(t) = k_a \times B(t) \times (K_N - IKKKa(t)) \times k_{a20}/(k_{a20} + A_{20}) - k_i \times IKKKa(t) \quad (1)$$

**IKK in neutral state (IKKn):** the first term describes recovery from the state IKKii (amount of which is  $IKKii = K_{NN} - IKKn - IKKa - IKKi$ ) second depletion due to activation by IKKKa (since IKK must be phosphorylated at two serine residues 177 and 181 the second order reaction is assumed):

$$\frac{d}{dt} IKKn(t) = k_4 \times (K_{NN} - IKKn - IKKa - IKKi) - k_1 \times IKKKa^2(t) \times IKKn(t) \quad (2)$$

**IKK in the active state IKKa:** The first term represents transition from IKKi to IKKa mediated by IKKKa, whereas the second term represents depletion of IKKa due to its transformation into inactive form IKKi mediated by A20 (since IKK must be phosphorylated at two serine residues 177 and 181 the second order reaction is assumed):

$$\frac{d}{dt} \text{IKKa}(t) = k_1 \times \text{IKKKa}^2(t) \times \text{IKKn}(t) - k_3 \times \text{IKKa}(t) \times (k_2 + \text{A20}(t))/k_2 \quad (3)$$

**IKK in the inactive state IKKi:** The first term corresponds to the formation of inactive IKKi from IKKa by A20 mediated inactivation, whereas the second term describes transformation into IKKii:

$$\frac{d}{dt} \text{IKKi}(t) = k_3 \times \text{IKKa}(t) \times (k_2 + \text{A20}(t))/k_2 - k_4 \times \text{IKKi}(t) \quad (4)$$

**Phospho-IkB $\alpha$ (IkB<sub>p</sub>):** The first term describes IkB $\alpha$  phosphorylation due to catalytic action of IKKa, the second term catalytic degradation of phosphorylated IkB $\alpha$ :

$$\frac{d}{dt} \text{IkB}_p(t) = a_2 \times \text{IKKa}(t) \times \text{IkB}(t) - t_p \times \text{IkB}_p(t) \quad (5)$$

**Phospho-IkB $\alpha$  complexed to NF- $\kappa$ B (NF $\kappa$ B|IkB<sub>p</sub>):** The first term describes IkB $\alpha$  phosphorylation (in complexes with NF- $\kappa$ B) due to the catalytic action of IKKa, the second term catalytic degradation of phosphorylated IkB $\alpha$  (NF- $\kappa$ B is recovered):

$$\frac{d}{dt} (\text{NF}\kappa\text{B}|\text{IkB}_p)(t) = a_3 \times \text{IKKa}(t) \times (\text{NF}\kappa\text{B}|\text{IkB})(t) - t_p \times (\text{NF}\kappa\text{B}|\text{IkB}_p)(t) \quad (6)$$

**Free cytoplasmic NF- $\kappa$ B:** The first two terms represents liberation of free NF- $\kappa$ B due to degradation of IkB $\alpha$  in (NF $\kappa$ B|IkB) complexes and its depletion due to formation of these complexes. The third term accounts for liberation of NF- $\kappa$ B due to degradation of phospho-IkB $\alpha$ . The last term describes transport of free cytoplasmic NF- $\kappa$ B to the nucleus:

$$\begin{aligned} \frac{d}{dt} \text{NF}\kappa\text{B}(t) = & c_{6a} \times (\text{NF}\kappa\text{B}|\text{IkB})(t) - a_1 \times \text{NF}\kappa\text{B}(t) \times \text{IkB}(t) + t_p \times (\text{NF}\kappa\text{B}|\text{IkB}_p)(t) \\ & - i_1 \times \text{NF}\kappa\text{B}(t) \end{aligned} \quad (7)$$

**Free nuclear NF- $\kappa$ B:** The first term describes transport into the nucleus. The second term represents depletion of free nuclear NF- $\kappa$ B due to the association with nuclear I $\kappa$ B $\alpha$  and is adjusted, by multiplying the synthesis coefficient  $a_1$  by  $k_v = V/U$  to the smaller nuclear volume:

$$\frac{d}{dt} \text{NF}\kappa\text{B}_n(t) = i_1 \times \text{NF}\kappa\text{B}(t) - a_1 \times k_v \times \text{I}\kappa\text{B}_n(t) \times \text{NF}\kappa\text{B}_n(t) \quad (8)$$

**A20 protein:** Described by its synthesis and constitutive degradation:

$$\frac{d}{dt} \text{A20}(t) = c_4 \times \text{A20}_t(t) - c_5 \times \text{A20}(t) \quad (9)$$

**A20 transcript:** The first term stands for NF- $\kappa$ B inducible synthesis, while the second term describes degradation of the A20 transcript:

$$\frac{d}{dt} \text{A20}_t(t) = c_1 \times G_{\text{A20}}(t) - c_3 \times \text{A20}_t(t) \quad (10)$$

Note that Eq. (10) (as well as Eq. 13) naturally produces saturation in transcription speed. When the nuclear amount of regulatory factor NF- $\kappa$ B is very large, then the binding probability is much larger than the dissociation probability, and the gene state would be  $G_{\text{A20}} = 2$  for most of the time. In such case the transcription would proceed at a maximum rate,  $2 c_1$ .

**Free cytoplasmic I $\kappa$ B $\alpha$  protein:** The first term accounts for IKK $\alpha$  induced phosphorylation, the second for NF- $\kappa$ B binding. The second line describes I $\kappa$ B $\alpha$  synthesis and the constitutive degradation of I $\kappa$ B $\alpha$ . The last two terms represent transport into and out of the nucleus:

$$\begin{aligned} \frac{d}{dt} \text{I}\kappa\text{B}(t) = & -a_2 \times \text{IKK}\alpha(t) \times \text{I}\kappa\text{B}(t) - a_1 \times \text{I}\kappa\text{B}(t) \times \text{NF}\kappa\text{B}(t) \\ & + c_4 \times \text{I}\kappa\text{B}_t(t) - c_{5a} \times \text{I}\kappa\text{B}(t) - i_{1a} \times \text{I}\kappa\text{B}(t) + e_{1a} \times \text{I}\kappa\text{B}_n(t) \end{aligned} \quad (11)$$

**Free nuclear I $\kappa$ B $\alpha$  protein:** The first term corresponds to I $\kappa$ B $\alpha$  association with nuclear NF- $\kappa$ B (adjusted, by multiplying the synthesis coefficient  $a_1$  by  $k_v = V/U$  for the smaller nuclear volume resulting in larger concentration), and the last two terms represent the transport into and out of the nucleus:

$$\frac{d}{dt} \text{IkB}_n(t) = -a_1 \times k_v \times \text{IkB}_n(t) \times \text{NFkB}_n(t) + i_{la} \times \text{IkB}(t) - e_{la} \times \text{IkB}_n(t) \quad (12)$$

**IkB $\alpha$  transcript:** The first term stands for NF- $\kappa$ B inducible synthesis, whereas the second term describes transcript degradation:

$$\frac{d}{dt} \text{IkB}_t(t) = c_1 \times G_{\text{IkB}}(t) - c_3 \times \text{IkB}_t(t) \quad (13)$$

**Cytoplasmic (NFkB|IkB) complexes:** The first two term describes formation of the complexes due to IkB $\alpha$  and NF- $\kappa$ B association and their degradation. The third term represents phosphorylation of (NFkB|IkB) complexes due to the catalytic activity of IKKa. The last term represents transport of the complex from the nucleus:

$$\begin{aligned} \frac{d}{dt} (\text{NFkB} | \text{IkB})(t) &= a_1 \times \text{IkB}(t) \times \text{NFkB}(t) - c_{6a} \times (\text{NFkB} | \text{IkB})(t) \\ &- a_3 \times \text{IKKa}(t) \times (\text{NFkB} | \text{IkB})(t) + e_{2a} \times (\text{NFkB} | \text{IkB}_n)(t) \end{aligned} \quad (14)$$

**Nuclear (NFkB|IkB $_n$ ) complexes:** Described by their formation due to IkB $_n$  and NF- $\kappa$ B $_n$  association adjusted, by multiplying the synthesis coefficient  $a_1$  by  $k_v = V/U$ , to the smaller nuclear volume resulting in larger concentration) and their transport out of the nucleus:

$$\frac{d}{dt} (\text{NFkB} | \text{IkB}_n)(t) = a_1 \times k_v \times \text{IkB}_n(t) \times \text{NFkB}_n(t) - e_{2a} \times (\text{NFkB} | \text{IkB}_n)(t) \quad (15)$$

**Extracellular TNF $\alpha$  concentration:** The concentration decreases due to natural degradation and binding to TNFR1 receptors:

$$\frac{d}{dt} \text{TNF}_{\text{ext}}(t) = -k_{\text{deg}} \times \text{TNF}_{\text{ext}}(t) \quad (16)$$

### Propensities of receptors and genes activation and inactivation

The receptors activate independently with activation propensity  $r_r^b(t)$  due to binding of extracellular ligands. Receptors inactivate independently with propensity  $r_r^d$  constant, due to dissociation of ligands or internalization of receptors:

$$r_r^b(t) = k_b \times \text{TNF}_{\text{ext}}(t); \quad r_r^d = k_d \quad (17)$$

We assume that both A20 and I $\kappa$ B $\alpha$  genes have two homologous copies independently activated due to NF- $\kappa$ B binding, and inactivated due I $\kappa$ B $\alpha$  mediated removal of NF- $\kappa$ B molecules, and that binding and dissociation propensities  $r^b(t)$  and  $r^d(t)$  respectively, are equal for each copy:

$$r^b(t) = q_1 \times \text{NF}\kappa\text{B}_n(t); \quad r^d(t) = q_2 \times \text{I}\kappa\text{B}_n(t) \quad (18)$$

The state of gene copy  $G^i$  ( $i = 1, 2$ ) is  $G^i = 1$  whenever NF- $\kappa$ B is bound to the promoter regulatory site, and  $G^i = 0$  when the site is unoccupied. As a result the gene state  $G = G^1 + G^2$  can be equal to 0, 1 or 2.

### Deterministic Approximation

In the deterministic approximation of the hybrid model the evolution of number of active receptors and of gene states are governed by the following deterministic equations:

**Number of active TNFR1 receptors B:** The first term describes receptor activation due to binding of TNF $\alpha$  trimers, while the second one describes their inactivation due to dissociation or internalization of receptors. For the sake of simplicity we assume that internalized receptors are replenish by the novo synthesis:

$$\frac{d}{dt} B(t) = k_b \times \text{TNF}_{\text{ext}}(t) \times (M - B) - k_d \times B \quad (19)$$

**The state of A20 and I $\kappa$ B $\alpha$  genes  $G_{A20}$  and  $G_{\text{I}\kappa\text{B}}$ :** The first term describes A20 or I $\kappa$ B $\alpha$  genes activation due to NF- $\kappa$ B binding, while the second one their inactivation due to removal of NF- $\kappa$ B molecules by I $\kappa$ B $\alpha$ :

$$\frac{d}{dt} G(t) = q_1 \times \text{NF}\kappa\text{B}_n(t) \times (2 - G) - q_2 \times \text{I}\kappa\text{B}_n(t) \times G \quad (20)$$

## Reporter gene kinetics

**The state of reporter gene:** We assume that all reporter genes (i.e. NF-κB responsive genes) have two homologous copies independently activated due to NF-κB binding, and inactivated due to spontaneous dissociation or IκBα mediated removal of NF-κB molecules, and that binding and dissociation propensities  $r_R^b(t)$  and  $r_R^d(t)$ , respectively, are equal for each copy:

$$r_R^b(t) = q_{1R} \times \text{NF}\kappa\text{B}_n(t); \quad r_R^d(t) = q_{2RR} + q_{2R} \times \text{I}\kappa\text{B}_n(t) \quad (21)$$

As a result reporter gene state  $G_R = G_R^1 + G_R^2$  can be equal to 0, 1 or 2 with respect to the number of activated copies.

**Reporter gene mRNA:** The first two terms describes NF-κB independent and dependent synthesis, while the last one is mRNA degradation:

$$\frac{d}{dt} R_t(t) = c_{1RR} + c_{1R} \times G_R - c_{3R} \times R_t(t) \quad (22)$$

As a result, the mRNA kinetics of any reporter gene is governed by 6 independent parameters (specific for a given gene);  $q_{1R}$ ;  $q_{2RR}$ ;  $q_{2R}$ ;  $c_{1RR}$ ;  $c_{1R}$ ;  $c_{3R}$ . The first three describe gene activation and inactivation kinetics, while the next three NF-κB independent and dependent synthesis and mRNA degradation. Most (but not all) of the mRNA time profiles observed in this study can be explained *only* by varying the mRNA degradation parameter  $c_{3R}$ .

## Numerical implementation for the stochastic model

In model computations, the amounts of all the substrates are expressed as the numbers of molecules. Since we use the ODEs to describe most of the model kinetics, amounts of molecules are not integer numbers, but since these numbers are in most cases much greater than 1, such description is reasonable. The numerical scheme implemented follows that of Lipniacki et al. (2007):

- (1) At simulation time  $t$ ; for given states  $G_{A20} = G_{A20}^1 + G_{A20}^2$ ,  $G_{I\kappa B} = G_{I\kappa B}^1 + G_{I\kappa B}^2$ , and  $G_R = G_R^1 + G_R^2$  of the A20, IκBα, and reporter genes, and number of active (bound)

receptors B (with M equal to the total number of receptors) we calculate the total propensity function  $r(t)$  of occurrence of any of the activation and inactivation reactions

$$r(t) = r_r^b \times (M - B) + r_{A20}^b(t) \times (2 - G_{A20}) + r_{I\kappa B}^b(t) \times (2 - G_{I\kappa B}) + r_R^b(t) \times (2 - G_R) \\ + r_r^d \times B + r_{A20}^d(t) \times G_{A20} + r_{I\kappa B}^d(t) \times G_{I\kappa B} + r_R^d(t) \times G_R \quad (23)$$

(2) We select two random numbers  $p_1$  and  $p_2$  from the uniform distribution on  $[0, 1]$ .

(3) Using the fourth order MATLAB solver we evaluate the system of ODEs accounting for fast reactions, until time  $t+\tau$  that:

$$\log(p_1) + \int_t^{t+\tau} r(s) ds = 0 \quad (24)$$

(4) There are 10 possible reactions:

- a) Receptors may be activated or inactivated. Typically, there are many inactive receptors which may be activated and active receptors which may be inactivated, but since the receptors are assumed to be identical it is not important which one of them changes its state.
- b) NF- $\kappa$ B may bind to or dissociate from any of two alleles of A20, I $\kappa$ B $\alpha$ , or reporter genes.

In this step we determine which one of 10 potentially possible reactions occurs at time  $t+\tau$  using the inequality

$$\sum_{i=1}^{k-1} r_i(t+\tau) < p_2 \times r(t+\tau) < \sum_{i=1}^k r_i(t+\tau) \quad (25)$$

where  $r_i(t+\tau)$ ,  $i=1, \dots, 10$  are individual reaction propensities and  $k$  is the index of the reaction to occur.

(5) Finally time  $t+\tau$  is replaced by  $t$  and we go back to item (1).



In all simulations, we simulate a resting cell for time  $t$  randomly chosen from the interval of 10 to 20 hours in order to get equilibrated and randomized initial conditions.

## Model fitting and parameters

The validation of the proposed model is based on our comprehensive single cell, microfluidic chamber experiments, and additionally on major experiments of other researchers that account for different aspects of NF- $\kappa$ B regulatory pathway kinetics:

### This study

- Single cell nuclear NF- $\kappa$ B temporal profiles for tonic TNF $\alpha$  stimulation (TNF $\alpha$  doses from 0.01 to 100 ng/nl)
- Fraction of responding cells versus TNF $\alpha$  dose

### Previous studies

- Detailed IKK activity profile (HeLa), Delhase et al. (1999)
- Wild type and A20 $^{-/-}$  cells, IKK activity, A20 mRNA, Nuclear NF- $\kappa$ B, (MEFs) Lee et al. (2000), see Fig. M6 for IKK activity and nuclear NF- $\kappa$ B profiles
- System responses to short TNF $\alpha$  pulses in MEFs, Hoffmann et al. (2002), Werner et al. (2008), see Fig. M3 for 1, 2, 5, 15 and 45 min.
- Short pulse-pulse experiments – recovery of IKK activity (HeLa), Lipniacki et al. (2007)
- Single cell pulse-pulse-pulse and tonic stimulation – Nuclear NF- $\kappa$ B measurements in SK-N-AS and HeLa (tonic stimulation only), Nelson et al. (2004), Ashall et al. (2009), see Figs. M4 and M5

When comparing the model predictions with other experiments one should keep in mind that some kinetic features of responses and NF- $\kappa$ B pathway are cell specific. Cell lines exhibit a substantial difference in sensitivity to stimulation; for example MEFs appear to be about 10 times more sensitive than 3T3 cells. 3T3 cells are more sensitive than HeLa cells that exhibit only one NF- $\kappa$ B peak under tonic stimulation. These differences may result from different numbers of TNFR1 receptors, IKKK or IKK levels – and the current knowledge do not allow to determine at which level of signal transduction the differences in cell sensitivity arise. Thus, for the sake of simplicity and to account for different cell lines sensitivity we assumed that cells from these lines

have different numbers of TNFR1 receptors; HeLa: 500; 3T3: 2000; SK-N-AS 5000; MEFs: 10000 receptors each.

The population studies of NF- $\kappa$ B by the groups of Baltimore, Levchenko and Hoffmann, or single cell experiments performed by White and colleagues were all performed in cell culture wells. The most important difference between such well and microfluidic chambers is the much larger media volume per cell in the wells, which determines the available number of molecules per cell at a given dose. As shown in Fig. M.2 the number of TNF $\alpha$ -TNFR1 complexes, depends not only on the initial concentration but also on the media volume per cell.

We do not fit the parameters to any given single experiment using automatic fitting procedures like the one proposed by Fajarewicz et al. (2007), but we intuitively choose the set of parameters which produces qualitative agreement with a major subset of existing data. Because of a large number of undetermined parameters, this is a tedious task, but in our opinion it is better to produce a model in qualitative agreement with the current and previous experiments, than a model perfectly fitted to a single experiment with limited set of data.

We based our choice of parameters on both single cell and population experiments. To compare our model with population based experiments, we average a large number of single-cell stochastic simulations. This procedure is much more time consuming than comparing the deterministic model with the population data, but as already shown in the case of low dose TNF $\alpha$  stimulation, the population data do not correspond to any biological process, and thus constructing the model fitted to such a data is not justified.

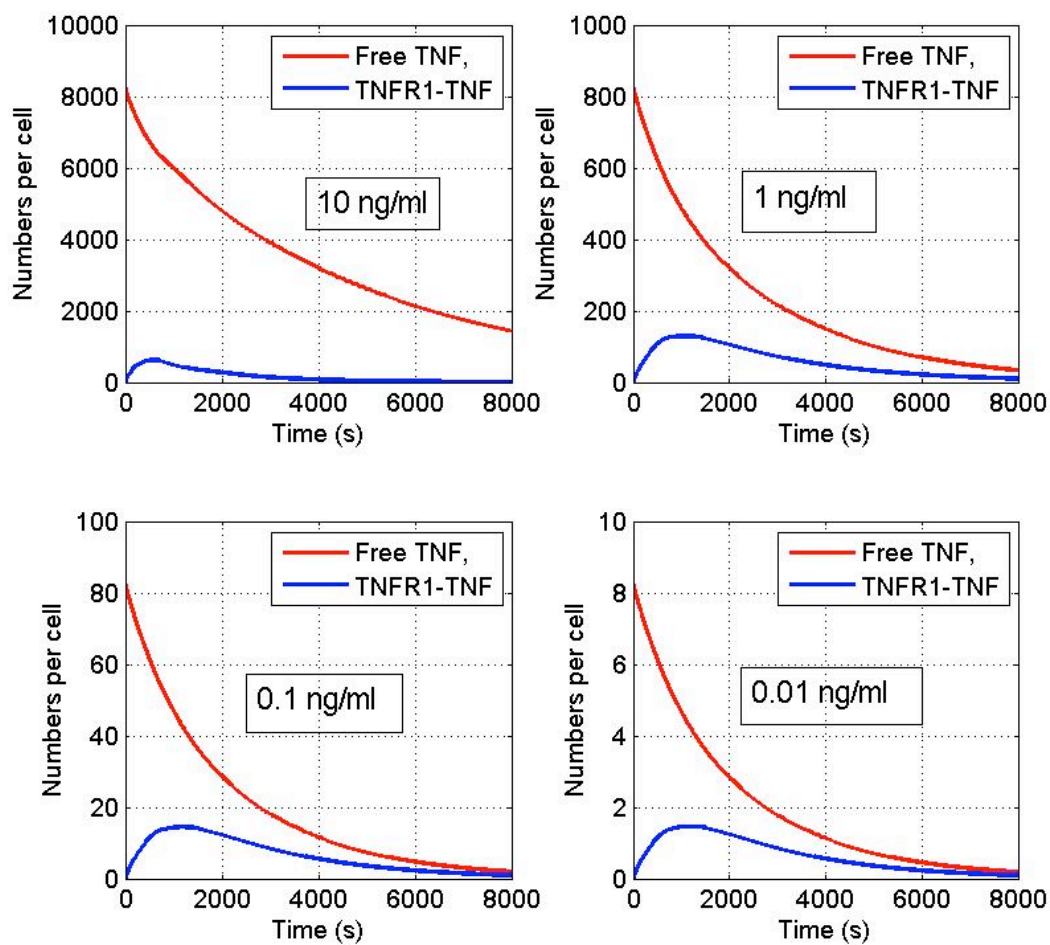
We applied the following method of choosing the parameter values:

- 1) Start from a reasonable set of parameters, which produces a correct steady state in the absence of TNF $\alpha$  signal.
- 2) Proceed with the signal initiated by downstream the autoregulatory loops.
- 3) Iterate item 2 until the fit to all the data is satisfactory.

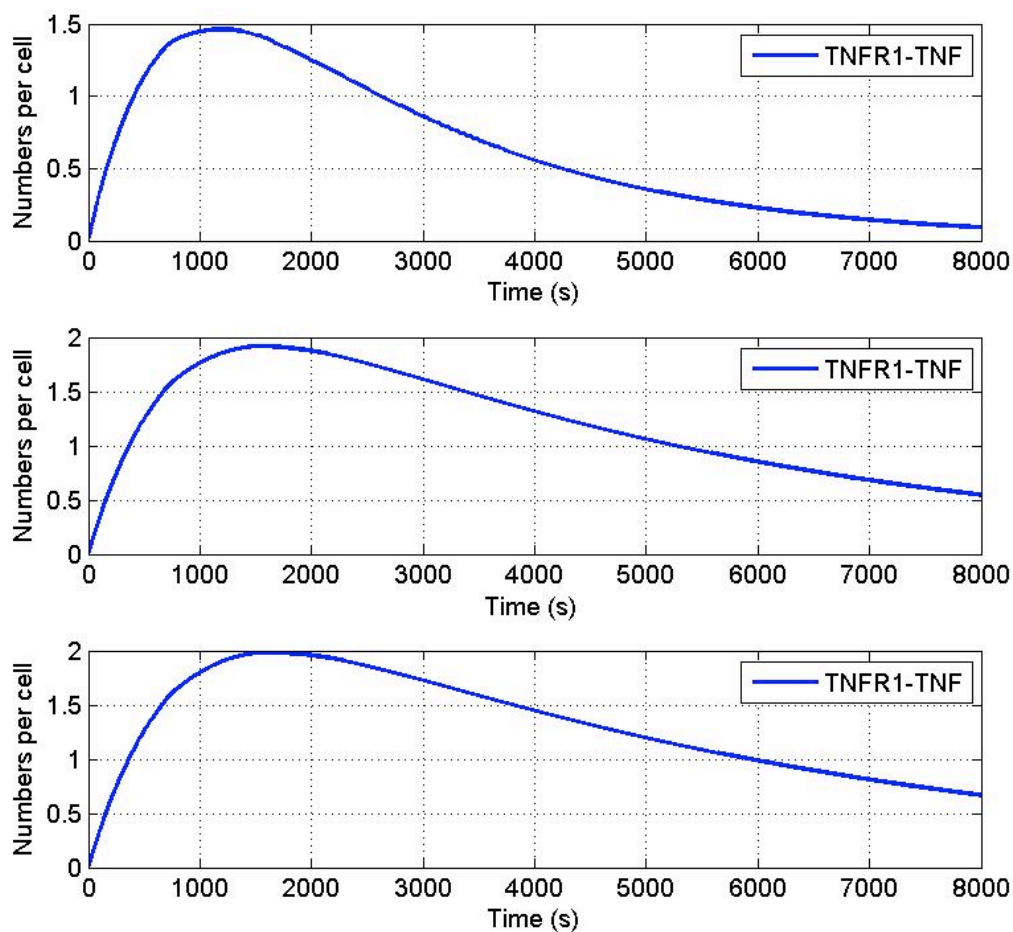
As stated in item 2, we first fit the coefficients regulating IKK activation using existing data on IKK activity, then the coefficients regulating degradation of the cytoplasmic (I $\kappa$ B $\alpha$ |NF- $\kappa$ B) and I $\kappa$ B $\alpha$  degradation, and so forth. It is necessary to iterate the signal tracing several times, until the

fit is satisfactory. We find that the set of parameters that produce a satisfactory fit is not unique. This ambiguity is mainly caused by the lack of measurements of absolute values of protein or mRNA amounts. The action exerted by some components of the pathway onto the rest of the pathway is determined by their amounts multiplied by undetermined coupling coefficients. Hence, once a good set of parameters is found, another one can also be produced using, for example, a smaller coupling coefficient and by proportionately enlarging the absolute level of the component. Since not all parameters may be determined based on existing data we have assumed values of part of parameters mostly based on our intuition and fitted the remaining ones. By such approach we show how much information can be inferred from available experimental data. Ambiguity in parameter determination leads to significant differences between parameters of our model and the corresponding parameters chosen by other groups of researcher. Values of all model parameters used in our model is listed in Table M2.

## FIGURES

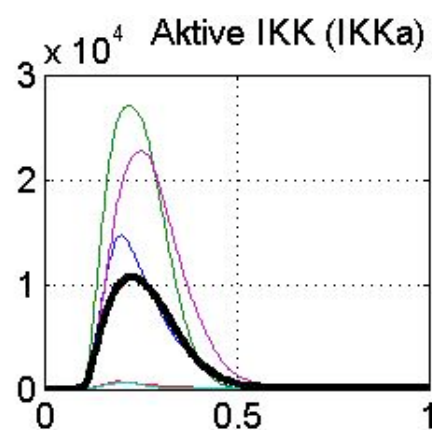
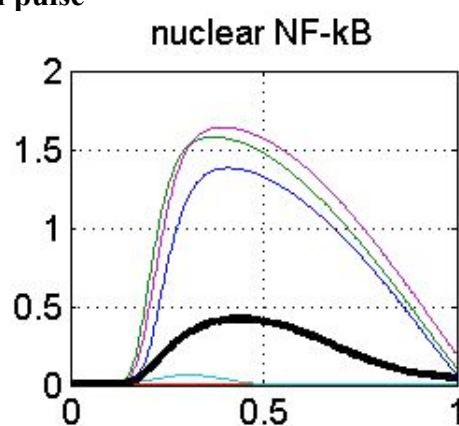


**Fig. M1** Number of free and bound TNF $\alpha$  molecules per cell for four initial TNF $\alpha$  concentrations: 10, 1, 0.1, and 0.01 ng/ml.

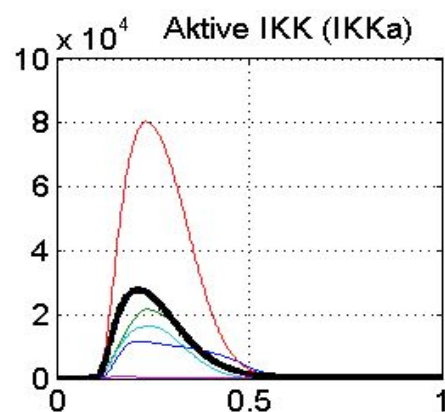
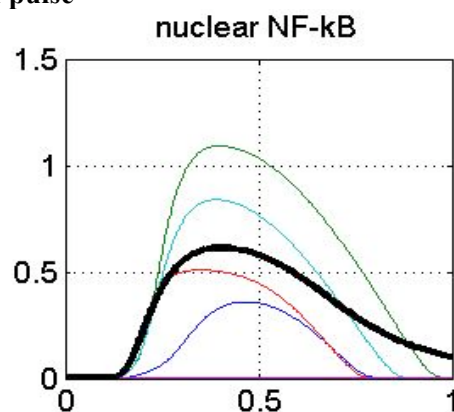


**Fig. M2** Number bound TNF $\alpha$  molecules per cell for initial TNF $\alpha$  concentration 0.01 ng/ml in the 35 nanoliter microfluidic chamber, and chambers having 10 times and 100 times larger volume but containing the same number of cells.

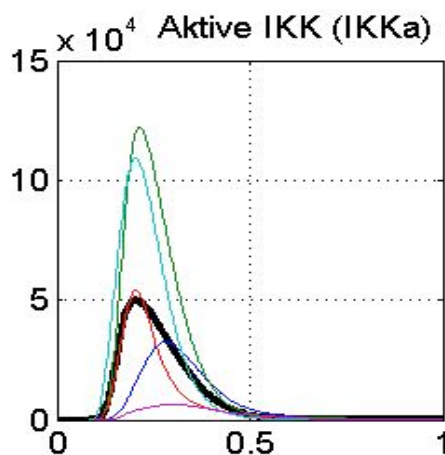
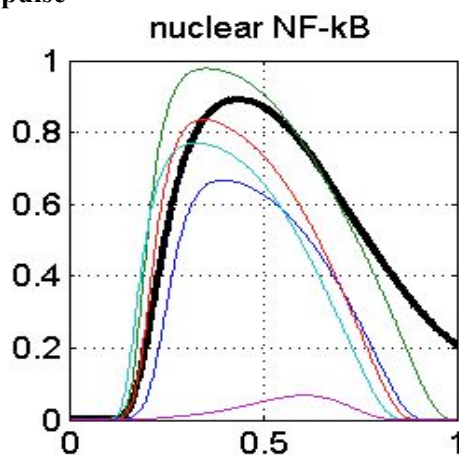
1 min pulse



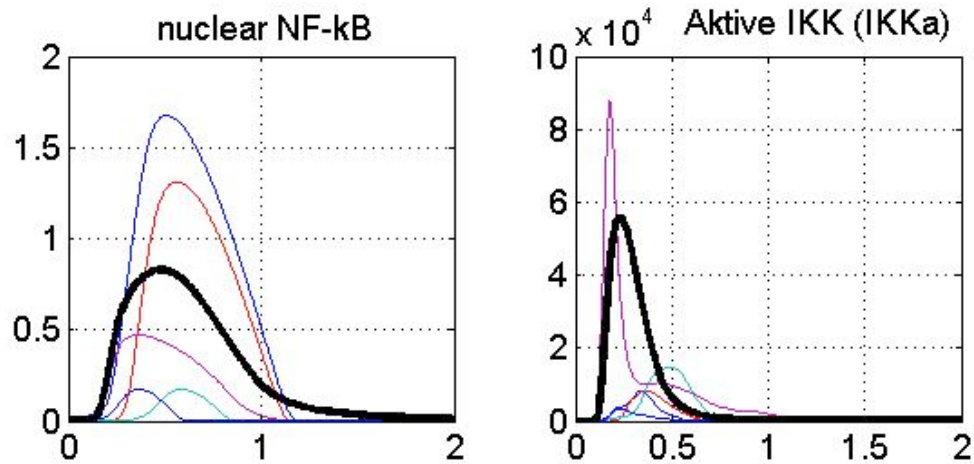
2 min pulse



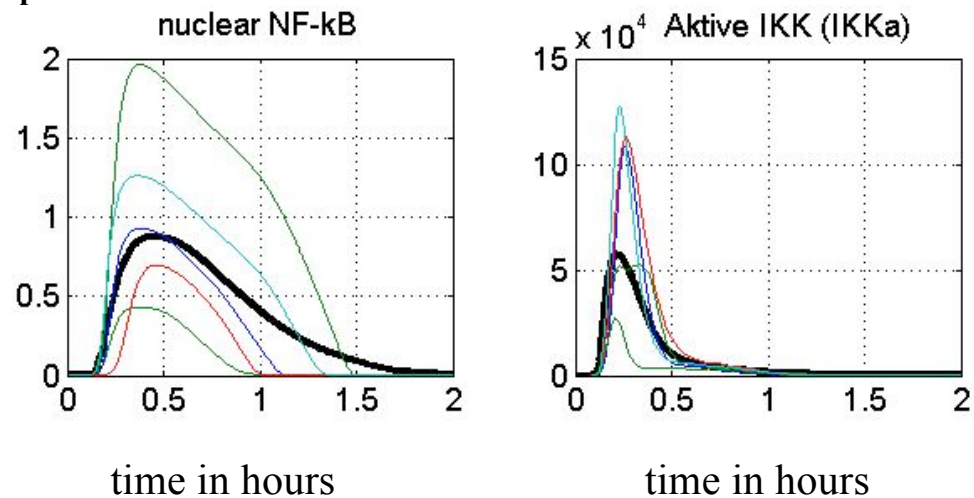
5 min pulse



## 15 min pulse

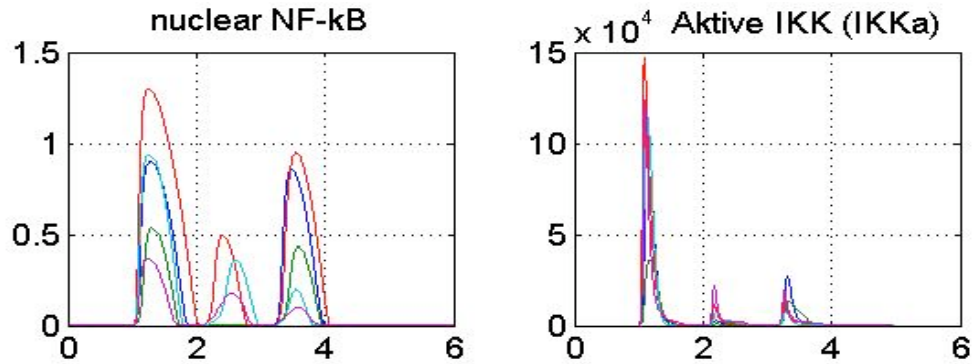
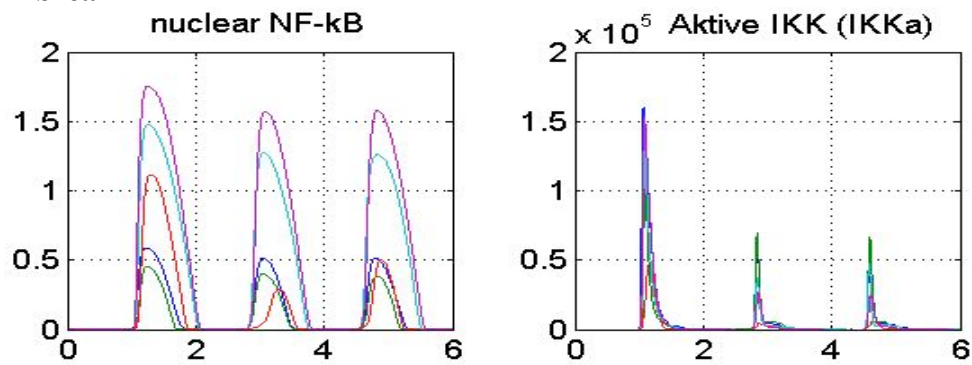
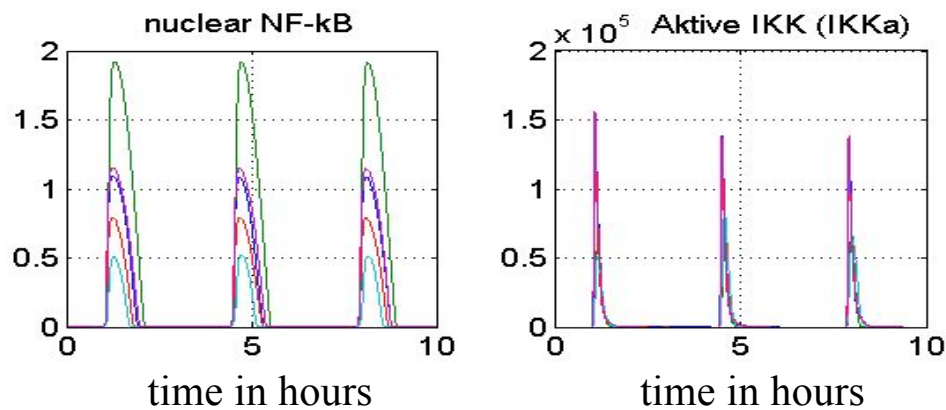


## 45 min pulse



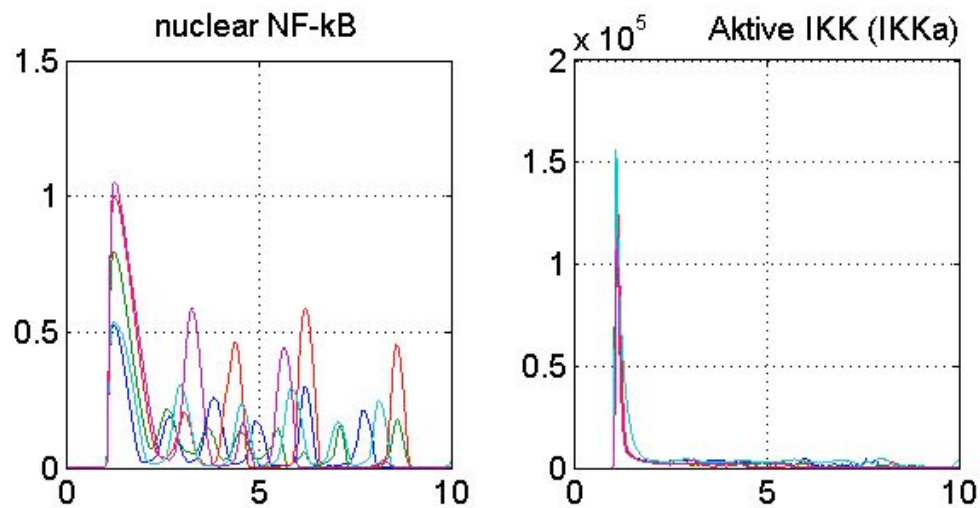
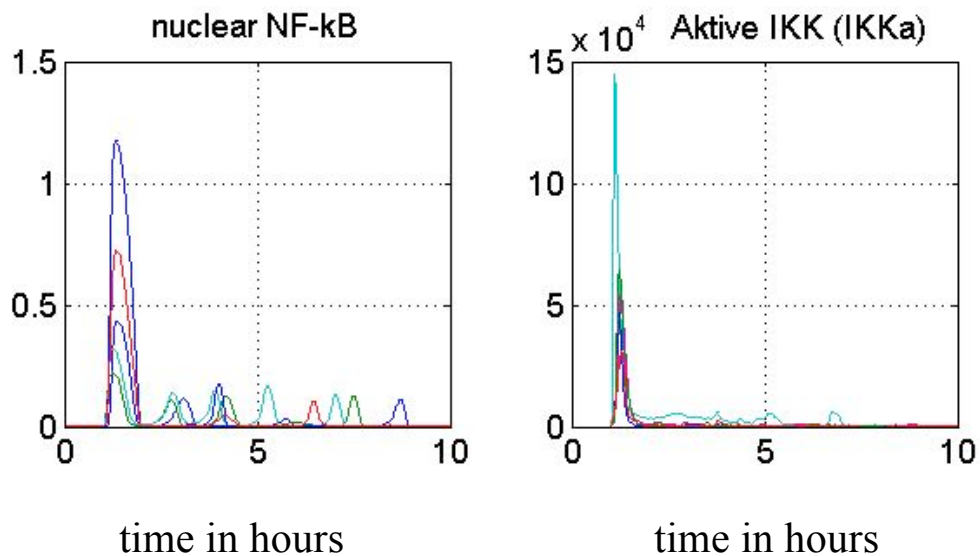
**Fig. M3** Numerical simulations of short-pulsed stimulations of MEFs (10000 TNFR1). Cells are stimulated by 1, 2 5, 15 and 45 minute long 1ng/ml TNF $\alpha$  pulses, Bold black line: average over 100 cells, thin lines: single cell stochastic trajectories. Nuclear NF- $\kappa$ B is given in  $10^5$  molecules. The 1 min long 1ng/pulse is sufficient to activate most of the cells, as observed by Werner et al. (2008). The responses to 15 and 45 min pulses are in agreement with Hoffman et al. 2002 experiment.



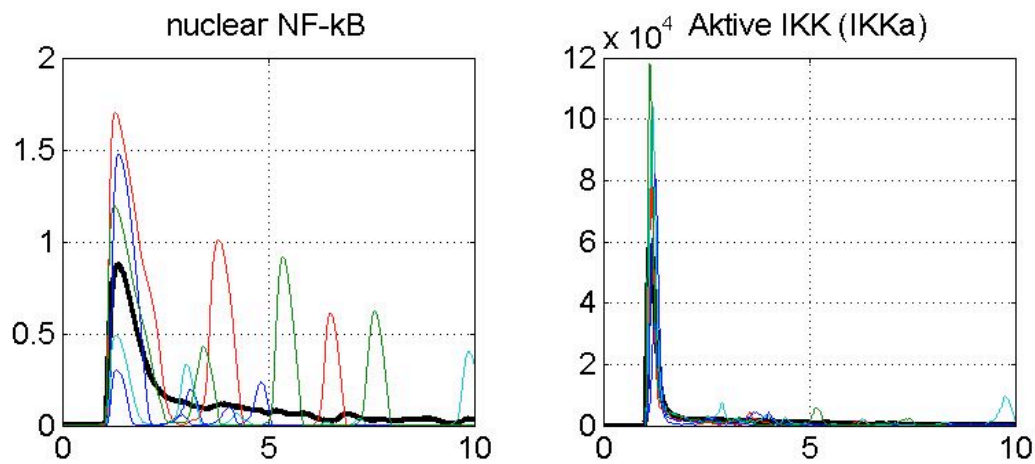
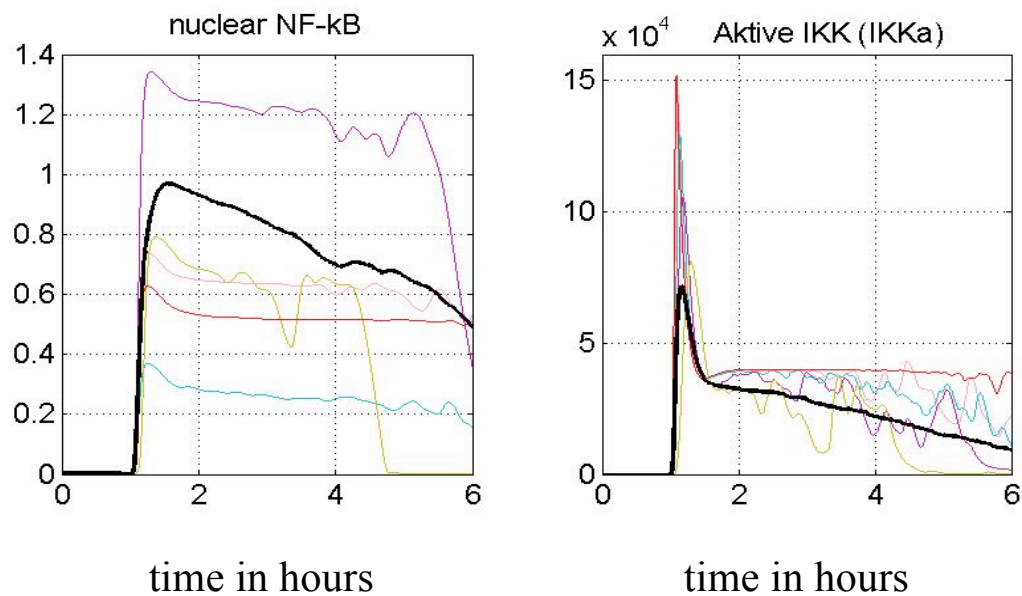
**60 min break****100 min break****200 min break**

**Fig. M4** Simulations of three consecutive short-pulses of  $\text{TNF}\alpha$  stimulation (Pulse-Pulse-Pulse) on SK-N-AS cells (5000  $\text{TNFR1}$ ). Cells were stimulated by three, five minutes long, 10ng/ml pulses separated by respectively 60, 100 min and 200 min breaks. Nuclear NF-kB is given in  $10^5$  molecules. The results are in good agreement with Ashall et al. (2009) and Lipniacki et al., (2007, IKK activity).



**SK-N-AS cells****HeLa cells**

**Fig. M5** Simulated tonic stimulation of SK-N-AS and HeLa cells by 10ng/ml TNF $\alpha$ , where each cell type is assumed to have 5000 and 500 TNFR1 receptors, respectively (see Nelson et al., 2004). Nuclear NF- $\kappa$ B is given in  $10^5$  molecules. The high sensitivity to TNF $\alpha$  in SK-N-AS cells results in multiple oscillations, while the low sensitivity in HeLa cancer cell line results in reduced NF- $\kappa$ B activity.

**Wild type cells****A20 -/- cells**

**Fig. M6** Active IKK and nuclear NF-κB profiles in wild type and A20 -/- MEFS (10000) under tonic TNFα 1ng/ml stimulation. Bold black line: average over 100 cells, thin lines: single cell stochastic trajectories. Nuclear NF-κB is given in 10<sup>5</sup> molecules.

**Table 3** Response to pulse-pulse TNF stimulation (20 min pulse – 180 min break – 20 min pulse). B – fraction of cell responding to both pulses, F – fraction of cells responding to first pulse only, S – fraction of cells responding to second pulse only. Cells in which peak of nuclear NF- $\kappa$ B exceeds  $0.2 \text{ NF-}\kappa\text{B}_{\text{tot}} = 2 \times 10^4$  are considered responding.

TNF dose	Type of model				Experiment
	deterministic	extrinsic noise only	intrinsic noise only	Full model	
0.05ng/ml	B=0.0% F=0.0% S=0.0%	B=2% F=1% S=0.0%	B=5% F=23% S=19%	B=6.5% F=10% S=7.5%	B=8% F=8% S=6%
0.1ng/ml	B=0.0% F=0.0% S=0.0%	B=7% F=2% S=0.0%	B=18% F=29% S=23%	B=12% F=12% S=11%	B=11% F=10% S=9%
0.2ng/ml	B=0.0% F=100.0% S=0.0%	B=16% F=4% S=0%	B=53% F=22% S=20%	B=22% F=14% S=13%	B=17% F=9% S=5%

In the deterministic model there exists threshold dose for cell activation,  $\text{Th1} \approx 0.2 \text{ ng/ml}$  above which cells respond to first peak only, and  $\text{Th2} \approx 0.25 \text{ ng/ml}$  above which cells respond to both pulses. Since the response to first peak results in elevated A20 and I $\kappa$ B $\alpha$  levels cells are slightly more resistant to the second pulse and thus  $\text{Th2} > \text{Th1}$ .

In the extrinsic noise model cell responses are highly correlated, there are very few cells responding to first peak only and none of cells respond to the second pulse only.

In the intrinsic noise model cell activation at both pulses is almost independent and thus

$$B \approx (F + B)(S + B)$$

## References

- Ashall, L. et al., Pulsatile stimulation determines timing and specificity of NF- $\kappa$ B-dependent transcription. *Science* 2009, **324**:242-246.
- Chan, F. K-M., Three is better than one: Pre-ligand receptors assembly in the regulation of receptors signaling *Cytokine* 2007, **37**:101-107.
- Covert, M.W., Leung, T.H., Gaston, J.E., & Baltimore, D., Achieving stability of lipopolysaccharide-induced NF- $\kappa$ B activation. *Science* 2005, **309**: 1854-1857.
- Delhase M, Hayakawa M, Chen Y, Karin M: Positive and negative regulation of I $\kappa$ B $\alpha$  kinase activity through IKK. *Science* 1999, **284**:309-313.
- Fujarewicz K, Kimmel M, Lipniacki T, Swierniak A, Adjoint systems for models of cell signaling pathways and yheir application to parameter fitting. *IEEE/ACM Transactions on*

Computational Biology and Bioinformatics 2007, **4**:322-335.

Grell M, Wajant H, Zimmermann G, Scheurich P: The type 1 receptor (CD120a) is the high-affinity receptor for soluble tumor necrosis factor. *Proc Natl Acad Sci USA* 1998, **95**:570-575.

Haseltine EL, Rawlings JB: Approximate simulation of coupled fast and slow reactions for stochastic chemical kinetics. *J Chem Phys* 2002, **117**:6959-6969.

Hoffmann A, Levchenko A, Scott ML, Baltimore D: The I $\kappa$ B $\alpha$  - NF- $\kappa$ B signaling module: Temporal control and selective gene activation. *Science* 2002, **298**:1241-1245.

Krikos A, Laherty CD, Dixit VM: Transcriptional activation of the tumor necrosis factor alpha-inducible zinc finger protein, A20, is mediated by kappa B elements. *J Biol Chem* 1992, **267**:17971-17976.

Lipniacki T, Paszek P, Brasier AR, Luxon B, Kimmel M: Mathematical model of NF- $\kappa$ B regulatory module. *J Theor Biol* 2004, **228**:195-215.

Lipniacki T, Paszek P, Brasier AR, Luxon B, Kimmel M: Stochastic regulation in early immune response, *Biophys J* 2006, **90**:725-742.

Lipniacki, T., Puszynski, K., Paszek, P., Brasier, A. R., Kimmel, M. Single TNF- $\alpha$  trimers mediating NF- $\kappa$ B activation: Stochastic robustness of NF- $\kappa$ B signaling. *BMC Bioinformatics* 2007, **8**:736-756.

Lee EG, Boone DL, Chai S, Libby SL, Chien M, Lodolce JP, Ma A: Failure to regulate TNF-induced NF- $\kappa$ B and cell death responses in A20-deficient mice. *Science* 2000, **289**:2350-2354.

Nelson DE, et al.: Oscillations in NF- $\kappa$ B signaling control the dynamics of gene expression. *Science* 2004, **306**:704-708.

Schomer-Miller B, Higashimoto T, Lee Y-K, Zandi E: Regulation of I $\kappa$ B $\alpha$  kinase (IKK) complex by IKK -dependent phosphorylation of the T-loop and C terminus of IKK. *J Biol Chem* 2006, **281**:15268-15276.

Werner SL, Barken D, Hoffmann A: Stimulus specificity of gene expression programs determined by temporal control of IKK activity. *Science* 2005, **309**:1857-1861.

Werner, S. L., Kearns, J. D., Zadorozhnaya, V., Lynch, C., O'Dea, E. L., Boldin, M. P., Ma, A., Baltimore, D., Hoffmann, A. Encoding NF- $\kappa$ B temporal control in response to TNF: distinct roles for the negative regulators I $\kappa$ B $\alpha$  and A20. *Genes and Development*, 2008, **22**:2093-2101

Wertz IE, O'Rourke, KM, Zhou H, Eby M, Aravind L, Seshagiri S, Wu P, Wiesmann C, Baker R, Boone DL, Ma A, Koonin EV, Dixit VM: De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF- $\kappa$ B signaling. *Nature* 2004, **430**:694-699.

Zhang SQ, Kovalenko A, Cantarella G, Wallach D: Recruitment of the IKK signalosome to the p55 TNF receptor: RIP and A20 bind to Nemo (IKK) upon receptor stimulation. *Immunity* 2000, **12**:301-311.