Theoretical analyses predict A20 regulates period of NF-κB oscillation

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Abstract. The nuclear-cytoplasmic shuttling of NF-κB is characterized by damped oscillations of the nuclear concentration with a time period of around 1-2 hours. The NF-κB network contains several feedback loops modulating the overall response of NF-κB activity. While IkBo is known to drive and IkBe is known to dampen the oscillations, the precise role of A20 negative feedback remains to be elucidated. Here we propose a model of the NF-κB system focusing on three negative feedback loops (IkBo, IkBe and A20) which capture the experimentally observed responses in wild-type and knockout cells. We find that A20, like IkBe, efficiently dampens the oscillations albeit through a distinct mechanism. In addition, however, we have discovered a new functional role of A20 by which it controls the oscillation period of nuclear NF-κB. The design based on three nested feedback loops allows independent control of period and amplitude decay in the oscillatory response. Based on these results we predict that adjusting the expression level of A20, e.g. by siRNA, the period can be changed by up to a factor 2.

Keywords: Nested Feedback Loops, Immune Response, Oscillations
Introduction

Nuclear Factor-kappa B, NF-κB, is a family of dimeric transcription factors involved in a number of important processes such as immune response, cellular growth and apoptosis [1]. NF-κB regulates the expression of more than a hundred genes and is implicated in a large number of diseases, including cancer, heart diseases and asthma [1]. Nuclear translocation of NF-κB, necessary for its activity, is triggered by a wide variety of stress signals: endotoxin LPS, cytokines IL-1 and the tumor necrosis factor (TNF). Fluorescence imaging of the TNF-triggered NF-κB activity in single mammalian cells shows distinct "spiky" but asynchronous oscillations in the level of nuclear NF-κB [2]; populations of mouse fibroblast cells continuously exposed to TNF exhibit damped and smooth – probably due to population averaging – oscillations in the nuclear NF-κB concentration. The production of damped oscillations with a time period of around 1.5 hours thus seems to be a robust characteristic of the NF-κB system. NF-κB is regulated by several negative feedback loops: two acting through the inhibitor proteins IκBα, ε which bind and sequester it in the cytoplasm. Another feedback regulates concentrations of nuclear NF-κB through A20, see Fig. 1. Addition of TNF activates the IκB kinase (IKK) which in turn causes the phosphorylation, and subsequent degradation, of the IκB inhibitor proteins, thus releasing NF-κB. Free NF-κB translocates to the nucleus inducing transcription of the inhibitor proteins, IκBε and IκBα, and A20. In turn the IκB proteins inhibit the NF-κB transcription factor by actively exporting it out of the nucleus. A20 on the other hand acts upstream by inactivating IKK, see Fig. 1A. There is another inhibitor protein, IκBβ, but it is only slightly induced by NF-κB compared to the two other inhibitor proteins [3] resulting in a weak negative feedback on NF-κB. We will thus omit it in our model, see Fig. 1B.

Each IκB protein forms a negative feedback loop as they are all transcriptionally activated by NF-κB. The loops are not identical. Knockout mutant studies in bulk indicate that in the absence of IκBε the nuclear NF-κB oscillations are enhanced whereas there are no oscillations in the absence of IκBα. It has been suggested that IκBε dampens oscillations generated by IκBα, which is consistent with the differences in their half-lives – IκBε is at least twice as stable as IκBα [4]. IκBα is activated almost instantly while IκBε activation occurs 37 min after NF-κB enters the nucleus. This difference in half life and time of activation allows us to define IκBα as a "fast" and IκBε as a "slow" negative feedback, see Fig. 1.

A20 feedback acts upstream of NF-κB and IκB. It is an important regulator of late IKK activity and was shown experimentally to be required for the drop in NF-κB activity separating early and late phase response to TNF when measured in bulk [4]. Cells deficient in A20 show persistent IKK activity and develop severe inflammation and cachexia [5].

The physiological importance of NF-κB transcription factor and its intriguing dynamical behavior made it a center of attention for decades both from an experimental and theoretical point of view [6 7].

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Figure 1. Schematic drawing of the core of NF-κB regulatory network. A) Details of the NF-κB regulation. TNF activates the IκB kinase (IKK) which in turn causes the phosphorlylation, and subsequent degradation, of the IκB inhibitor proteins, thus releasing NF-κB. Free NF-κB translocates to the nucleus inducing transcription of the inhibitor proteins, IκBε and IκBα, and A20. The IκB proteins inhibit the NF-κB transcription factor by actively exporting it out of the nucleus. A20 acts upstream by inactivating IKK. The IκBβ is not shown. B) Nested negative feedback perspective on the NF-κB regulation. NF-κB is regulated by two parallel negative feedbacks acting through the inhibitor proteins IκBα, ε and A20 negative feedback, acting upstream by inactivating IKK. The variation in the greyscale of the three feedback loops indicates the difference in timescales: dark grey stands for fast and light grey for slowest.

pathway was proposed in Hoffmann et. al [8] and used to understand the dynamical responses of the NF-κB wild-type and IκB knockout, e.g. oscillations and their absence in knockouts. This model has later been modified and used by Nelson et al [2] to analyze oscillations in single cells. Krishna et al 2006 [9] showed that the model can be significantly reduced while still capturing the essential dynamical features, in particular showing spiky oscillations in single cells.

Both modeling and experimental results suggest that A20 is important for lowering the level of nuclear NF-κB after TNF stimulus [5, 4]. Other studies have focused on details of where and how A20 acts in the pathway [10]. To address the discrepancy between bulk and single cell data Ref. [11, 10] introduced stochasticity and showed that averaging single cell stochastic dynamics leads to a smooth damped response in bulk.

In the current view of the system, IκBα feedback drives the oscillations and the
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Figure 2. Model simulations of the four states of the cell obtained by numerical integration of the Ordinary Differential Equations describing the model in Methods section. A: wild-type, B: \( \text{IkB}\varepsilon^{-/-} \), C: \( \text{A}20^{-/-} \), D: \( \text{A}20^{-/-}/\text{IkB}\varepsilon^{-/-} \). In each panel blue line is the concentration of active IKK, red is the normalized concentration of the nuclear NF-κB and dashed green line is the concentration of total inhibitor proteins, \( I_t = \text{IkB}\alpha + \text{IkB}\varepsilon \). Note the differences in NF-κB oscillation period and decrease in the amplitude in absence of A20 and \( \text{IkB}\varepsilon^{-/-} \) and increased levels of IKK in late stage for A20 knockout cells.

Figure 3. A20 changes the period of nuclear NF-κB oscillations. WT is in black, reproduced from Figure 2A, and A20 knockout is in red, reproduced from Figure 2C. Note the difference in steady states after oscillations are damped.

IkB\varepsilon feedback dampens the oscillations. From the bulk experiments A20 is known to lower the level of nuclear NF-κB after stimulus. It is not known how the temporal profile of NF-κB in single cells is affected by A20 [4]. We would like to investigate whether and how A20 is modifying NF-κB oscillatory behavior in single cells. To address this question we will extend the NF-κB model by Krishna et al. [9] to include IkB\varepsilon and A20 negative feedbacks.
**Model**

Figure 1 shows schematic representation of the model. It contains three negative feedback loops centered around NF-κB: IκBα, IκBε and A20.

The dynamical variable of most importance is $N_n$, the nuclear NF-κB concentration. The first term in the equation for $N_n$ is the rate of increase in nuclear NF-κB concentration due to import of free NF-κB from the cytoplasm. This rate is lower for higher levels of the IκB proteins. The other two negative terms model the decrease of the nuclear concentration due to sequestration by the IκBs and subsequent export into the cytoplasm. Over the timescales we are interested in there is no significant production or degradation of NF-κB. The mRNA levels of IκBα and IκBε are regulated through a sigmoidal function of NF-κB, given by $\frac{N^2}{N^2+K^2}$. Here we assumed that there is a weak cooperativity in NF-κB activating transcription of IκBα/ε with Hill coefficient two. We also include a small basal level of transcription in IκBα, which has little influence on NF-κB dynamics but has an important role in reproducing the IκBα and IκBε mRNA fold induction experimentally measured in [3].

At the protein level, the rate of protein increase is linearly proportional to the respective mRNA. The rate of decrease in IκBα is controlled by IKK-independent degradation, $I_\alpha/\tau_\alpha$ and IKK-dependent degradation. The rate of IκBα decay is proportional to both IKK and the concentration of complexes formed between IκBα and cytoplasmic NF-κB, $N_c \propto IKK[IκBa : N_c]$. Assuming that reaction rates for complex formation are much faster then the nuclear import/export and IκBα degradation, the concentration of $[IκBa : N_c]$ can be derived to be $(1 - N_n)/(1 + IκB\varepsilon + IκBα)$ (see Supplementary Materials for more details). The changes in the IκBε protein levels are governed by the same terms as for IκBα, but with different rate constants, which overall make the negative feedback through IκBε slower and weaker (see Supplementary Material for details). Apart from this, the equations for IκBε are the same as for IκBα.

The IκB Kinase (IKK) is the driving force of the system as its activation leads to the degradation of the inhibitor proteins, the IκBs, and thereby the release of NF-κB. IKK is activated upon stimulation of the membrane receptor but the detailed mechanism for this activation remains to be clarified. We have chosen to use the mechanism earlier proposed in Ref. [10] to model the IKK activation: a three step process where IKK is converted from its neutral state to being active by the triggering signal, TNF, see Fig. 1A. The active IκB kinase can turn itself off and go back into neutral state before being activated again by the TNF signal. The IκB kinase is shut down by A20 which inhibits the transformation from inactive to neutral IKK thereby leaving IKK in an inactive state, see Fig. 1A.

The first 30 minutes of IKK adaption-like temporal profile in response to TNF stimulation – which appears to be independent of A20 regulation – is modeled such that IKK peaks after about 15 minutes and then goes to a new steady state after 30 minutes of TNF induction. The new steady state is however determined by A20: it is high in the absence of A20 and decreases with increasing concentrations of A20. This is a slow
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**Figure 4.** Analysis of the IκBε−/− and A20 feedback loops. The strength of each loop is varied by varying the IκBε transcription and the A20 coupling to IKK. The white circle represents the reference state of wild type (Figure 2A). The color-coded are: A) The average period taken between the 2nd and 3rd peak. B) The amplitude of NF-κB averaged over 2nd and 3rd peak with a lower boundary of 1%. C) The dampening effect, tells how fast are oscillations damped, measured by difference in amplitude between the 2nd and 4th peak. Bifurcation line is shown in black and separates regions of sustained oscillations from regions of damped oscillations. This bifurcation line has been found by counting the number of peaks in the nuclear concentration of NF-κB within the simulated timespan (see Methods section for details). Both IκBε transcription and A20 coupling to IKK is able to change the amplitude (B) and dampen the oscillations (C) but only the A20 coupling to IKK is able to alter the period of the oscillations (A).

feedback as IKK must first activate NF-κB leading to the production of A20 that in turn shuts down the pathway.

We have taken most rates and timescales from existing literature wherever possible and manually adjusted so that the model reproduces the following experimental observations:

1. Wild-type cells show damped oscillations in nuclear NF-κB with a time period of 60-100 min.
2. Mutants with IκBα alone show enhanced oscillations with same period of 60-100 min, while those with IκBε alone do not show oscillations.
3. The fold induction in mRNA of IκBε reaches twice the level of fold induction of the IκBα mRNA

**Results and discussion**

**Model validation**

The basic response of our model, with the default parameters, to a continuous presence of TNF is damped oscillations of nuclear NF-κB. The original wild type response as well as IκBε and IκBα knockout matches the experimental observations (see Fig. 2A, B), Supplementary Fig. S1 and interactive applet[12]. The time period of the oscillations is about 100 minutes, which also matches experiments. Thus, the basic response (criterion 1 and 2) is correctly reproduced by the model, Fig. 2. We also checked the model against criteria 3 and other known knockouts, for further details see Supplementary material.
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A20 changes the period of nuclear NF-κB oscillations

![Figure 5](image)

**Figure 5.** Set of characteristic responses in nuclear NF-κB obtained at different IκBε transcription rates and A20 coupling strengths to IKK. The color coded is the average period taken between the 2nd and 3rd peak (a section of A in Fig. 4). Several parameter sets have been selected both within and outside of the bifurcation border (the black line). Both IκBε transcription and A20 coupling to IKK is able to change the amplitude and dampening of the nuclear transcription factor activity but only the A20 coupling to IKK is able to alter the period of the oscillations.

It has been suggested that A20 lowers nuclear NF-κB levels of the late phase of the response. This observation stems from bulk experiments [13, 4]. However, the role of A20 has not been investigated in single cells where NF-κB has oscillatory response [10]. Using the proposed model we aimed to investigate the role of A20 as a modifier of the NF-κB oscillatory behavior. We find that A20 is able to adjust the period of nuclear NF-κB oscillations in the range from 1 to 3 hours. Below we describe the details of our finding.

We have modeled the NF-κB response in A20 knockout cells and were surprised to find that not only is the resting level of the late phase of the NF-κB response increased (as shown in Fig. S2) but also the oscillations become more pronounced when compared to wild-type cells, see Fig. 2 A,C and Fig. 3. The amplitude of the NF-κB response is decreased in the presence of A20 whereas the period is increased. This effect of A20 dampening the oscillations and increasing the period is even more visible in IκBε knockout cells where the oscillations are sustained, compare Fig. 2B and D.

A20 only has an effect on the temporal profile of IKK in its late phase: the A20 mRNA level peaks at 30 min and the protein shows its effect after 45-60 min [13, 4]. This feature is captured by the model where the level of IKK in the late phase is pushed
Theoretical analyses predict A20 regulates period of NF-κB oscillation down in the presence of A20 and generates low frequency NF-κB oscillations. In the absence of A20 the late phase of IKK stays at a high level and generates high frequency oscillations, compare Fig. 2A and C and Fig. 3.

*IκBε does not change the period of NF-κB oscillations*

Is A20 feedback loop dispensable and can IκBε have similar effect on the oscillations period? There are two possible mechanisms for IκBε to dampen IκBo oscillations: a) destructive interference, where IκBε oscillates on the same time scale as IκBo but with the shifted phase compared to IκBo, such phase shift or delay has been observed experimentally and b) where IκBε varies on a slower time scale and the interference between fast changing IκBo and slow IκBε results in damped oscillations. In order for a) to be true IκBε and IκBo must exhibit comparable strength and frequency and thus IκBo knockout should oscillate in single cells, however at present there is no such data available and bulk experiments show no oscillations in the IκBo knockout. On the other hand it is known that IκBε has a slower degradation through IKK dependent mechanism which makes IκBε change on a slower time-scale. Furthermore the b) scenario can easily reproduce bulk data for IκBo knockout and therefore we model IκBε through scenario b). With this constraint IκBε does not influence the frequency in our model (compare 2A and B).

*Function of A20 and IκBε is robust to variation in parameter values*

Our observation that A20 decreases the frequency of NF-κB oscillations whereas IκBε is not capable of the same effect, is based on the model with specific set of parameters that were chosen to fit experimental results. To check if our observation is not merely a result of a specific parameter combination we have investigated how the period, amplitude as well as the oscillation dampening change with varying some of the model parameters. In Figure 4A we show how the period changes as the strengths of A20 and IκBε feedback loops vary 100 fold above and below the values we used in Figure 2. The period is almost independent of the strength of IκBε whereas it gradually increases as the strength A20 feedback loop increases, thus supporting our conclusion that A20 is a key regulator of the frequency of NF-κB oscillations. It is important to note that although it is possible to decrease the frequency by increasing the strength of the IκBε loop when IκBε transcription rate is larger than 50 (see top left corner in Figure 4A), the resulting oscillations are strongly damped as illustrated in Figure 4C.

*Reasons for the differential effect of A20 and IκBε on the period*

We can understand why A20 and not IκBε feedback can effectively adjust the frequency by looking at the IKK dependent degradation of IκBo and IκBε terms:

\[ \text{IκBo degradation} : \sim \frac{[N_c][I_a]}{1 + [I_a] + [I_e]} \]
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\[ I_κB_ε \text{ degradation} : \sim [K] \frac{[N_c][I_e]}{1 + [I_a] + [I_e]} \]

IKK acts similarly on both IκBε and IκBα feedbacks: Higher levels of IKK correspond to faster degradation of IκBε/α and thus shorter period. In case of IκBε, however, – because of competitive binding to cytoplasmic NF-κB – higher levels of IκBε will lead to faster degradation of IκBε but slower degradation of IκBα, see equations above. Thus IκBε can only increase the period when oscillations are driven by IκBα, whereas IKK can work in both directions. There are, however, limits to how much IKK can increase the period. This limit is set by the fact that as A20 increases, and IKK decreases, the system passes through a Hopf bifurcation (shown by black line in Figure 4) where it goes from sustained to damped oscillations so that the amplitude and period of oscillations are strongly correlated: the longer the period the smaller the amplitude, compare Figure 4A and B.

Conclusions

Growing evidence indicates that temporal control of NF-κB and the downstream genes is of crucial importance for cell functioning: constitutively active NF-κB is a cause of many human tumors. Active NF-κB turns on the expression of genes that keep the cell proliferating and protect the cell from conditions that would otherwise cause it to die via apoptosis. At the same time defects inactivating NF-κB result in increased susceptibility to apoptosis leading to increased cell death. It appears that the original solution to this dilemma is through transient activation of NF-κB [8] which on a single-cell level presents as damped oscillations. Such temporal control can allow for selective gene activation [13, 10]. Given that the NF-κB temporal response is of a high importance and is primarily regulated by three negative feedback loops we investigated the role of each negative feedback in shaping the response with the main focus on A20 negative feedback.

We find that the design of having two IκB feedback loops on the same level as NF-κB and the upstream feedback from A20 allows for a wide variety in possible NF-κB outputs. The fast feedback from IκBα generates the oscillatory behavior of the transcription factor which is damped by the delayed and out of phase activity of IκBε. This general response of NF-κB upon TNF stimulation is altered by the upstream feedback from A20 which is slower than the two other feedbacks and acts on the IκB kinase and not on the transcription factor itself. By changing the IKK profile, A20 is able to modulate the frequency of the transcription factor in a way not possible from the two IκB feedbacks. Additionally, A20 lowers the end level of the nuclear NF-κB meaning that when the system comes to rest more NF-κB is removed from the nucleus, in the presence of A20, compare Fig. 2 A and C and Fig. 3 [4]. (This is because IKK is less active and thus not degrading the IκBs as fast, leaving the inhibitor proteins to enter the nucleus and export the transcription factor).
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If the biphasic response – as seen in bulk experiments – is a result of the population average of single cells with oscillating NF-kB, then, in bulk experiments, A20 will exhibit its effect by affecting the timing of the second phase onset. Thus a specific prediction would be that – as the timing between first two peaks is shorter, see Supplementary Figure S3, – the second phase should start earlier in A20 knockout cells.

We found that similarly to IκBε, A20 is able to dampen oscillations albeit through a different mechanism. Whereas IκBε dampens oscillations through competition with IκBo for binding to NF-κB and acts about an hour after TNF induction, A20 dampens oscillations by inhibiting active IKK during late phase of the response, after about 2-3 hours. A distinct novel finding of our investigation suggests that not only does A20 dampen the oscillations, it can also adjust the period of the oscillations. Thus, A20 together with IκBε allows independent tuning of both dampening – through IκBε negative feedback – and the frequency – through A20 negative feedback. This combination of nested feedback loops covers a wider variety of temporal responses where one can access both sustained oscillations and damped oscillations with low or high frequency.

Our findings lead to a clear prediction that in single cells decreasing the coupling between A20 and IKK should lead to higher frequency oscillations in NF-κB. This can be experimentally tested by knocking down A20 with siRNA. Our model also predicts that the frequency of oscillations can be further increased by increasing the TNF dose in A20 knockout cells, where the IKK level in the late phase of the response is proportional to TNF dose. An interesting future direction would be to examine how the diversity of NF-κB oscillating temporal profiles created by nested feedback loops can allow for selective gene activation.

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Methods

Model Description

\[
\frac{dN_n}{dt} = \frac{A(N_t - N_n)}{(K_I + \eta I_\alpha + \eta I_\varepsilon)} - B(I_\alpha + I_\varepsilon)\frac{N_n}{(\delta + N_n)}
\] (1)

\[
\frac{dI_{mA}}{dt} = p + t_a\frac{N_n^2}{(K_D^2 + N_n^2)} - \gamma_{ma}I_{mA}
\] (2)

\[
\frac{dI_\alpha}{dt} = k_{ila}I_{mA} - \alpha_\alpha K_I\frac{(N_t - N_n)I_\alpha}{(K_I + \eta I_\alpha + \eta I_\varepsilon)} - \gamma_\alpha I_\alpha
\] (3)

\[
\frac{dI_{me}}{dt} = t_e\frac{N_n^2}{(K_D^2 + N_n^2)} - \gamma_{me}I_{me}
\] (4)
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\[
\frac{dI_\varepsilon}{dt} = k_{tie}I_{ma} - \alpha \varepsilon K \left(\frac{(N_t - N_n)I_{\varepsilon}}{K_I + \eta I_\alpha + \eta I_{\varepsilon}}\right) - \gamma \varepsilon I_{\varepsilon} \tag{5}
\]

\[
\frac{dA_m}{dt} = t_A N_n^2 - \gamma A_m A_m \tag{6}
\]

\[
\frac{dA}{dt} = k_{A}A_m - \gamma A A \tag{7}
\]

\[
\frac{dK}{dt} = T(K_t - K - K_i) - \mu K^2 \tag{8}
\]

\[
\frac{dK_i}{dt} = \mu K^2 - \beta \frac{K_i}{(\sigma A^2 + 1)} \tag{9}
\]

\[
\frac{dA}{dt} = k_{\Delta}A_m - \gamma A A \tag{7}
\]

The equations are all rescaled, see Supplementary Materials for details of the rescaling and the parameter values.

**Approximating Bifurcation Line**

To find the border for the bifurcation we counted the number of peaks in the nuclear concentration of NF-κB. We counted the oscillations as sustained if the number of peaks was 100 or above. This approximation slightly overestimates the size of the region with sustained oscillations and is not affecting any results and conclusions.
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| Variable      | Description                      |
|---------------|----------------------------------|
| $N_n$         | nuclear NF-κB                    |
| $I_{α/ε}$     | free IκB                          |
| $I_{mα/ε}$    | IκB mRNA                         |
| $A_m$         | A20 mRNA                         |
| $K_i$         | inactive IKK                      |
| $K_i$         | inactive IKK                      |

| Parameter     | Description                                      | Value               |
|---------------|---------------------------------------------------|---------------------|
| B             | proportionality factor of the export of nuclear NF-κB | 102.6               |
| A             | proportionality factor of the import of NF-κB      | 0.004               |
| η             |                                                   | 0.092               |
| δ             | concentration at which half of the IκBα/ε is bound in complex with NF-κB | 0.0414 μM           |
| p             | NF-κB in-dependent transcription rate of IκBα mRNA | $3.36 \times 10^{-8} \text{ min}^{-1}$ |
| $t_a$         | NF-κB dependent transcription rate of IκBα mRNA    | 0.0042 μM min$^{-1}$ |
| $t_c$         | NF-κB dependent transcription rate of IκBε mRNA    | 0.084 μM min$^{-1}$ |
| $t_A$         | NF-κB dependent A20 transcription rate             | 0.0168 μM$^{-1}$min$^{-1}$ |
| $tl_a$        | translation rate of IκBα                          | 0.0672 min$^{-1}$   |
| $tl_c$        | translation rate of IκBε                          | $1.2 \times 10^{-5}$ min$^{-1}$ |
| $tl_A$        | translation rate of A20                           | 0.3024 min$^{-1}$   |
| γ$I_{mα}$     | half-life of IκBα mRNA                           | 0.0168 min$^{-1}$   |
| γ$I_{mε}$     | half-life of IκBε mRNA                           | 0.00168 min$^{-1}$  |
| γ$I_{α/ε}$    | half-life of the IκB’s                           | 0.005 min$^{-1}$    |
| γ$A_{20m}$    | half-life of the A20 mRNA                         | 0.00168 min$^{-1}$  |
| γ$A_{20}$     | half-life of the A20                              | 0.00168 min$^{-1}$  |
| α$α$          | IKK dependent degradation of IκBα                 | 0.00025 min$^{-1}$  |
| α$ε$          | IKK dependent degradation of IκBε                 | 0.03-0.00025 min$^{-1}$ |
| μ              | rate of IKK self-inactivation                      | 0.063 min$^{-1}$    |
| σ              | strength of A20 negative feedback                 | 0.25                |

Table 1. Model variables and parameters

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Supplementary Materials

|        | WT       | $I_\varepsilon^{-/-}$ | $I_\alpha^{-/-}$ |
|--------|----------|-----------------------|------------------|
| $I\kappa B$ mRNA, fold | ![Graph](WT_IKB_mRNA.png) | ![Graph](IE_IKB_mRNA.png) | ![Graph](IA_IKB_mRNA.png) |
| $I\kappa B$ protein | ![Graph](WT_IKB_pro.png) | ![Graph](IE_IKB_pro.png) | ![Graph](IA_IKB_pro.png) |
| $A20$ | ![Graph](WT_A20.png) | ![Graph](IE_A20.png) | ![Graph](IA_A20.png) |

**Figure 1.** Model simulations of the four states of the cell: A: wild-type, B: $I\kappa B_\varepsilon^{-/-}$, C: $A20^{-/-}$, D: $A20^{-/-}/I\kappa B_\varepsilon^{-/-}$
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**Figure 2.** Model simulating the wild-type cell. Left column: Recordings of the durations of the 1st, 2nd and 3rd period measures as the time between first and second peak, second and third and third and fourth peak. Right column: Amplitude of 1st, 2nd and 3rd peak.

**Initial Model for IκBα regulation**

We use the following abbreviations: $N_n&N$, free nuclear and cytoplasmic NF-κB; $I_m$, IκB mRNA; $I_n&I$, free nuclear and cytoplasmic IκB; $(NT)_n&(NT)$, nuclear and cytoplasmic NF-κB-IκB complex; IKK, IκB kinase.

The equations for IκBα are the same as for IκBα so we just use variable $I$ in our
Theoretical analyses predict A20 regulates period of \( \text{NF-\kappa B} \) oscillations. The seven-variable model is defined by the equations

\[
\frac{dN_n}{dt} = k_{Nin}N - k_{fn}N_nI_n + k_{bn}(NI)_n,
\]

\[
\frac{dI_m}{dt} = k_I N^2 - \gamma_m I_m,
\]

\[
\frac{dI}{dt} = k_{it}I_m - k_f NI + k_b(NI) - k_{in}I + k_{out}I_n,
\]

\[
\frac{dN}{dt} = -k_f NI + (k_b + \alpha)(NI) - k_{Nin}N,
\]

\[
\frac{d(NI)}{dt} = k_f NI - (k_b + \alpha)(NI) + k_{Nout}(NI)_n,
\]

\[
\frac{dI_n}{dt} = k_{in}I - k_{out}I_n - k_{fn}N_nI_n + k_{bn}(NI)_n,
\]

\[
\frac{d(NI)_n}{dt} = k_f N_nI_n - (k_{bn} + k_{Nout})(NI)_n.
\]

**Reduced Model for \( \text{IKBs} \) regulation**

First, taking note of the fact that \( k_f \) and \( k_{fn} \) are large, we assume that all complexes are in equilibrium, i.e.

\[
k_f NI \approx (k_b + \alpha)(NI),
\]

\[
k_{fn}N_nI_n \approx (k_{bn} + k_{Nout})(NI)_n.
\]

Simulations show that these are good approximations. In terms of \( I^\text{tot}_n \equiv I_n + (NI)_n \) and \( N^\text{tot}_c \equiv N + (NI) = N_{\text{tot}} - N_n \), which are slowly varying, we can rewrite the above equations as follows:

\[
(NI) = (N_{\text{tot}} - N_n) \frac{I}{K_I + I},
\]

\[
N = (N_{\text{tot}} - N_n) \frac{K_I}{K_I + I},
\]

\[
(NI)_n = I^\text{tot}_n \frac{N_n}{K_N + N_n},
\]

\[
I_n = I^\text{tot}_n \frac{K_N}{K_N + N_n},
\]

where \( K_I \equiv (k_b + \alpha)/k_f = 0.035 \mu\text{M} \) and \( K_N \equiv (k_{bn} + k_{Nout})/k_{fn} = 0.029 \mu\text{M} \), using the parameter values above.

Using these expressions, the equations of the seven-variable model reduce to the following four (Fig. 9):

\[
\frac{dN_n}{dt} = k_{Nin}K_I \frac{(N_{\text{tot}} - N_n)}{K_I + I} - k_{Nout} \frac{I^\text{tot}_n N_n}{K_N + N_n},
\]
Theoretical analyses predict A20 regulates period of NF-κB oscillation

\[ \frac{dI_m}{dt} = k_I N_n^2 - \gamma_m I_m, \]
\[ \frac{dI}{dt} = k_{II} I_m - \frac{(N_{tot} - N_n)I}{K_I + I} - k_{Iin} I + k_{Iout} K_N \frac{I_{n}^{\text{tot}}}{K_N + N_n}, \]
\[ \frac{dI_{n}^{\text{tot}}}{dt} = k_{Iin} I - k_{Iout} K_N \frac{I_{n}^{\text{tot}}}{K_N + N_n} - k_{N_{in}} I_{n}^{\text{tot}} - N_n \frac{I_{n}^{\text{tot}}}{K_N + N_n}. \]

First, we note that the terms \(-k_{Iin} I\) and \(k_{Iout} K_N \frac{I_{n}^{\text{tot}}}{K_N + N_n}\) in the \(dI/dt\) equation are much smaller than \(-\alpha \frac{(N_{tot} - N_n)I}{K_I + I}\) and can be neglected as long as IKK is nonzero. Second, simulations reveal that the term \(k_{N_{in}} I_{n}^{\text{tot}}\), in the \(dI_{n}^{\text{tot}}/dt\) equation, also shows sharp spikes as a function of time which coincide with the spikes of \(N_n\). The value of this term is substantial only when \(N_n \gg K_n\), i.e., during the spikes of \(N_n\), and at those times \(I_{n}^{\text{tot}}\) dips to its minimum. We therefore make the approximation that \(I_{n}^{\text{tot}}\) can be replaced by its minimum value, \(I_{n,\text{min}}^{\text{tot}}\), which satisfies the equation

\[ k_{Iin} I = k_{Iout} K_N \frac{I_{n,\text{min}}^{\text{tot}}}{K_N + N_n} + k_{N_{in}} I_{n,\text{min}}^{\text{tot}} - N_n \frac{I_{n,\text{min}}^{\text{tot}}}{K_N + N_n}. \]

In the regime where \(N_n \gg K_n\) this gives

\[ I_{n,\text{min}}^{\text{tot}} \approx \frac{k_{Iin}}{k_{N_{in}}} I. \]

Using this we can reduce to a three-variable model

\[ \frac{dN_n}{dt} = k_{N_{in}} K_I \frac{(N_{tot} - N_n)}{K_I + I} - k_{Iin} I \frac{I N_n}{\delta + N_n}, \]
\[ \frac{dI_m}{dt} = k_I N_n^2 - \gamma_m I_m, \]
\[ \frac{dI}{dt} = k_{II} I_m - \alpha \frac{(N_{tot} - N_n)I}{K_I + I}. \]

**Parameters and variables re-scaling**

We start with the following system of equations

\[ \frac{dN_n}{dt} = k_{im} K_I \frac{(N_t - N_n)}{(K_I + I + I)} - B(I_{\alpha} + I_{\varepsilon}) \frac{N_n}{(\delta + N_n)} \quad (11) \]
\[ \frac{dI_{\alpha}}{dt} = p + t_{a} \frac{N_n^2}{(K_D^2 + N_n^2)} - \gamma_m I_{\alpha} \quad (12) \]
\[ \frac{dI_{\varepsilon}}{dt} = k_{ta} I_{\alpha} - \alpha_K I_{\alpha} \quad (13) \]
\[ \frac{dI_{m\alpha}}{dt} = k_{ta} I_{\alpha} - \alpha_K I_{\alpha} \quad (14) \]
\[ \frac{dI_{m\varepsilon}}{dt} = k_{te} I_{\alpha} - \alpha_K I_{\alpha} \quad (15) \]
Theoretical analyses predict A20 regulates period of NF-κB oscillation

\[
\frac{dA_m}{dt} = t_A N_n^2/K_D A - \frac{A_m}{\gamma_{Am}} \tag{.16}
\]
\[
\frac{dA}{dt} = k_{ti} A_m - \frac{A}{\gamma_A} \tag{.17}
\]
\[
\frac{dK}{dt} = T(K_t - K - K_i) - \mu K^2 \tag{.18}
\]
\[
\frac{dK_i}{dt} = \mu K^2 - \beta \frac{K_i}{\sigma A^2 + 1} \tag{.19}
\]

where \(k_{im}\) is the import of the NfκB into the nucleus, \(K_I\) is dissociation constant for IkBs binding to Nfκb, ...

Re-define

1. Re-define \(N_n\) by scaling with \(K_D\) and \(N_t\)

\[N'_n = N_n/K_D, \Rightarrow N'_t = N_t/K_D, \delta' = \delta/K_D, \alpha'_\alpha = \alpha_A/K_D, \alpha'_\varepsilon = \alpha_\varepsilon/K_D\]

and furthermore,

\[N''_n = N'_n/N'_t = N_n/N_t, \delta'' = \delta'/N'_t = \delta/N_t\]

After this transformations and redefining \(N''_n = N_n, \delta'' = \delta, etc.\) the equations will be rescaled to:

\[
\frac{dN_n}{dt} = k_{im} K_I \frac{(1 - N_n)}{(K_I + I_\alpha + I_\varepsilon)} - B(I_\alpha + I_\varepsilon) \frac{N_n}{(\delta + N_n)}
\]

\[
\frac{I_{ma}}{dt} = p + t_a \frac{N_n^2}{(1 + N_n^2)} - \gamma_{ma} I_{ma}
\]

\[
\frac{dI_\alpha}{dt} = k_{ta} I_{ma} - \alpha_A K \frac{(1 - N_n) I_\alpha}{K_I + I_\alpha + I_\varepsilon} - \frac{I_\alpha}{\gamma_\alpha}
\]

the equations for \(I_\varepsilon\) and \(I_{me}\) scaled similarly and the rest remains as above.

2. Re-define \(I_\alpha\) and \(I_\varepsilon\) by scaling with \(K_I\).

\[I'_\alpha/\varepsilon = \frac{I_\alpha/\varepsilon}{K_I} \Rightarrow B' = BK_I, \quad k'_{ta/\varepsilon} = k_{ta/\varepsilon}/K_I, \alpha'_\alpha/\varepsilon = \alpha_\alpha/\varepsilon/K_I\]

3. Re-define \(I_{am}\) and \(I_{em}\) by scaling with \(k_{ta}\).

Re-defining \(I'_{a/\alpha} = k'_{ta/\alpha} I_{a/\alpha} = k_{ta/\alpha}/K_I I_{a/\alpha}, \Rightarrow \ p' = pk_{ta/\alpha}/K_I, t'_a/\alpha = t_a/k_{ta/\alpha}/K_I\) results in following equations for

\[
\frac{dN_n}{dt} = k_{im} \frac{(1 - N_n)}{(1 + I_\alpha + I_\varepsilon)} - B(I_\alpha + I_\varepsilon) \frac{N_n}{(\delta + N_n)}
\]

\[
\frac{I_{ma}}{dt} = p + t_a \frac{N_n^2}{(1 + N_n^2)} - \gamma_{ma} I_{ma}
\]

\[
\frac{dI_\alpha}{dt} = I_{ma} - \alpha_A K \frac{(1 - N_n) I_\alpha}{1 + I_\alpha + I_\varepsilon} - \frac{I_\alpha}{\gamma_\alpha}
\]
Theoretical analyses predict A20 regulates period of NF-κB oscillation

4 Re-define $A_m$ by scaling with $k_{tlA}$.

$$A'_m = k_{tlA}A_m, \Rightarrow t'_A = k_{tlA}t_A/K_{DA}, \sigma = \sigma/k_{tlA}^2$$ thus equations for A20 become

$$\frac{dA_m}{dt} = t_A N_n^2 - \frac{A_m}{\gamma A_m}$$

$$\frac{dA}{dt} = A_m - \frac{A}{\gamma A}$$

5 Re-define $K$ and $K_i$ by scaling with $K_t$.

$$K = K/K_t, \quad K_i = K_i/K_t, \mu = K_t\mu$$ thus equations for IKK become

$$\frac{dK}{dt} = T(1 - K - K_i) - \mu K^2$$

$$\frac{dK_i}{dt} = \mu K^2 - \frac{\beta K_i}{\sigma A^2 + 1}$$

Thus the final system of equations is:

$$\frac{dN_n}{dt} = k_{im} \frac{(1 - N_n)}{(1 + I_{\alpha + I_{\varepsilon}})} - B(I_{\alpha + I_{\varepsilon}}) \frac{N_n}{(\delta + N_n)}$$

$$(.21)$$

$$\frac{dI_{\alpha}}{dt} = I_{\alpha} - \alpha_K \frac{(1 - N_n)I_{\alpha}}{1 + I_{\alpha} + I_{\varepsilon}} - \frac{I_{\alpha}}{\gamma_{\alpha}}$$

$$(.22)$$

$$\frac{dI_{\varepsilon}}{dt} = I_{\varepsilon} - \alpha_K \frac{(1 - N_n)I_{\varepsilon}}{1 + I_{\alpha} + I_{\varepsilon}} - \frac{I_{\varepsilon}}{\gamma_{\varepsilon}}$$

$$(.23)$$

$$\frac{dA_m}{dt} = t_A N_n^2 - \frac{A_m}{\gamma A_m}$$

$$(.24)$$

$$\frac{dA}{dt} = A_m - \frac{A}{\gamma A}$$

$$(.25)$$

$$\frac{dK}{dt} = T(1 - K - K_i) - \mu K^2$$

$$(.26)$$

$$\frac{dK_i}{dt} = \mu K^2 - \frac{\beta K_i}{\sigma A^2 + 1}$$

$$(.27)$$

and the scaled variables and parameters are:

$$N_n = \frac{N_n}{K_t}; \quad I_{\alpha/\varepsilon} = \frac{I_{\alpha/\varepsilon}}{K_t}; \quad I_{\alpha/\varepsilon} = \frac{k_{i\alpha/\varepsilon}}{K_t}; \quad A_m = k_{tlA}A_m; \quad K = \frac{K}{K_t}; \quad K_i = \frac{K_i}{K_t}$$

$$\delta = \frac{\delta}{K_t} = 0.0414; \quad B = BK_t = 0.014; \quad k_{tlA} = \frac{k_{tlA}}{K_t}; \quad p = \frac{p}{K_t}; \quad p = 58.4;$$

$$t_{a/e} = \frac{t_{a/e}}{K_t}, t_a = 7300, t_e = 27.4; \quad \alpha'_{\alpha/\varepsilon} = \frac{\alpha_{\alpha/\varepsilon}}{K_t}, \alpha_{\alpha} = 219, \alpha_{\varepsilon} = 6.57;$$

$$t_A = k_{tlA} \frac{t_A}{K_{DA}} = 18; \quad \sigma = \frac{\sigma}{K_t} = 77x10^{-5}; \quad \beta = 5; \quad \mu = K_t\mu = 100; \quad$$ TNF changes from 0.001 to 2.5; $K_i = 6.67$
Theoretical analyses predict A20 regulates period of NF-κB oscillation

| Variable | Description |
|----------|-------------|
| \( N_n = \frac{N_n}{N_t} \) | nuclear NF-κB normalized to total NF-κB |
| \( I_{α/ε} = \frac{I_{α/ε}}{K_I} \) | free IkBs scaled with dissociation constant \( K_I \) of IkBs binding to NF-κB |
| \( I_{ma/ε} = \frac{k_{1la/e}I_{ma/ε}}{K_I} \) | re-defined value of IkB mRNA, \( k_{1la/e} \) is the translation rate of IkBα/ε |
| \( A_m = k_{1lA}A_m \) | re-defined A20 mRNA, \( k_{1lA} \) is the A20 translation rate |
| \( K = \frac{K}{K_I} \) | active IKK normalized to the total IKK, \( K_t \) |
| \( K_i = \frac{K}{K_I} \) | inactive IKK normalized to the total IKK, \( K_i \) |

| Scaled Parameter | Description |
|------------------|-------------|
| \( δ = \frac{δ}{N_I} (\mu M^{-1}) \) | concentration at which half of the IkBα/ε is bound in complex with NF-κB, normalized to total NF-κB |
| \( B = BK_I \) | proportionality factor of the export of nuclear NF-κB, scaled with the respective translation rates and dissociation constant of NF-κB binding to IkBs, \( K_I \) |
| \( A = AK_I \) | proportionality factor of the import of NF-κB, scaled with the respective translation rates and dissociation constant of NF-κB binding to IkBs, \( K_I \) |
| \( p = \frac{p}{k_{1la/e}K_I} \) | constitutive NF-κB dependent transcription rate of IkBα mRNA, scaled with the respective translation rates and \( K_I \) |
| \( t_{a/e} = \frac{t_{a/e}}{k_{1la/e}K_I} \) | NF-κB dependent transcription rates of IkBs mRNA scaled with \( K_I \) |
| \( t_A = \frac{k_{1lA}T_A}{K_{DA}} \) | A20 transcription rate scaled with A20 translation rate, \( k_{1lA} \), and dissociation constant of NF-κB binding to DNA at the operator site controlling A20 promoter |
| \( γ_{1ma}/γ_{1ma} \) | half-life of IkBα mRNA scaled with \( γ_{1ma} \) |
| \( γ_{1me}/γ_{1ma} \) | half-life of IkBε mRNA scaled with \( γ_{1ma} \) |
| \( γ_{1α/e}/γ_{1ma} \) | half-life of the IkBα mRNA scaled with \( γ_{1ma} \) |
| \( γ_{A20m}/γ_{1ma} \) | half-life of the A20 mRNA scaled with \( γ_{1ma} \) |
| \( γ_{A20}/γ_{1ma} \) | half-life of the A20 scaled with \( γ_{1ma} \) |
| \( α_{α/ε} = \frac{α_{α/ε}}{K_DI} \) | rate constant for IKK dependent degradation scaled with dissociation constant of NF-κB binding to operator site at the IkB’s promoter, \( K_D \) and \( K_I \) |
| \( μ = K_i μ \) | rate of IKK self-inactivation scaled with total IKK, \( K_t \) |
| \( σ = \frac{σ}{k_{1lA}} \) | strength of A20 negative feedback scaled with the square of A20 translation rate, \( k_{1lA}^2 \) |