Modeling Interaction Patterns
at the Levels of Proteins, Cells and Humans

PhD Thesis

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Preface and Acknowledgements

Inspired work is not done single-handed and interaction and collaboration with other people is essential. This work was performed at Center for Models of Life (CMoL) at the Niels Bohr Institute, Copenhagen from April 2008 through March 2010. I thank Prof. Mogens Høgh Jensen and Prof. Kim Sneppen for giving me the opportunity to join the group of CMoL as a PhD student. Prof. Mogens Høgh Jensen has been my primary supervisor and I have very much enjoyed our work together. We have both scientifically and socially had many good times at the Niels Bohr Institute and during traveling. Sandeep Krishna has been involved in almost all my projects and has been a great support with his good ideas and sharp thinking. Ala Trusina joined my original project on NF-κB modelling at a time when new energy and encouragement was much appreciated. I am very grateful to Mogens, Sandeep and Ala for the supervision they have given me.

One year of the PhD training period was spent in the Signaling Systems Biology Laboratory at University of San Diego in the group of Prof. Alexander Hoffmann, during which I continued my primary focus on the NF-κB signalling system, now with an experimental perspective. I thank Alexander Hoffmann for hosting me at UCSD and his lab for welcoming me and especially Shannon Werner for teaching me experimental techniques for cell culturing and basic biochemistry. However, I did do a large part of my experiments in the laboratory of Dr. Gentry Patric under very talented supervision by Stevan Djakovic who taught me cloning and immunohistochemistry. I very much enjoyed the time I spent in both labs and I am grateful to all for welcoming me and taking up the challenge of opening the experimental world to a computational person. Part of my stay in San Diego was focused on transcription factor - DNA interaction in close collaboration with Prof. Elizabeth Komovies and students from her group.

After returning from San Diego I have been involved in two very interesting projects together with Szabolcs Semsey. We have worked together with Simone Pigolotti and Vedran Sekara on interaction between cells in a tissue and the effect of mutations. In a second project together with Lilla Sipos from Department of Psychology, Budapest, Hungary we have worked on a very interesting project on mother - infant interaction patterns. I very much appreciate the work and collaboration with them all.

The daily life at the b-floor, including students from the c-floor has been a great factor for motivation, including tea-breaks, inspiring interaction and support all through the process. This is a unique property of CMoL and the environment at the Niels Bohr Institute. Inspiration is, however, not only found at the office and I thank Tune Pers for stimulating and supporting discussion in our spare time together.

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Abstract

This thesis represents four studies at three different levels of interaction: protein-protein and protein-DNA interaction of the transcription factor NF-κB, lateral inhibition between cells in a tissue and a study of human mother-infant interaction.

The first study centers around the mammalian stress response system, the NF-κB pathway. NF-κB is found in most mammalian cells and is a key regulator of our immune response. Nuclear shuttling of the transcription factor has been observed on both single cell level and in bulk upon TNF activation, but the role of these oscillations are not well understood. Here we analyze the properties of oscillations of the nuclear-cytoplasmic shuttling of NF-κB generated by one fast feedback of the inhibitor protein IκBα nested within a slower feedback of A20 and alike proteins. We find that the nested feedback loops allow for a refined control of the oscillation period. Additionally, we elaborate on the potential ability for genes to decode oscillations.

The inhibitor protein IκBα is known to regulate the nuclear activity of NF-κB by export. In an independent study we consider the interaction between NF-κB and the DNA and find that IκB is not only exporting the transcription factor from the nucleus to the cytoplasm but is also mediating the actual dissociation of NF-κB from the DNA. This effect is found to be especially important in the presence of large amounts of non-specific DNA, where IκBα is responsible for the fast dynamics and low basal levels of nuclear NF-κB.

Secondly, we have looked at the interaction between cells in a tissue; how patterns in cell activity are formed in a tissue during early development. We have simulated the growth of tissues through four different mechanisms and tested their robustness to mutations. We find that patterns of ordered cell activity are more robust, and that mutations have a larger effect at the boundary of unstable areas. Mutations are additionally found to affect more neighboring cells when a silent cell is mutated active rather than when an active cell is silenced.

Finally, the fourth project takes place at the level of human interactions. We have used network analysis to decode high resolution mother-infant interaction data in preterm and full-term children. Comparison of preterm and full-term behavior shows that interactions with preterm children are often less harmonious. We find that mothers of preterm infants are periodically at two extremes of maladaptive behavior: they are either intrusive or disengaged. The pairing of an oversensitive infant with an intrusive/disengaged mother may place the infant at risk for later emotional, cognitive and behavioral disturbance.
Dansk Resumé

Denne afhandling repræsenterer fire studier af interaktion, der foregår på tre forskellige niveauer fra protein-protein og protein-DNA interaktion af transcriptionsfaktoren NF-κB over celler i et væv til menneskelig interaktion i samspelet mellem en mor og hendes barn.

Det første studie drejer sig om pattedyrs stressrespons, NF-κB-signalvejen. NF-κB findes i stort set alle pattedyrs celler og er en af hovedaktørerne i vores immunforsvar. Bevægelse af transcriptionsfaktoren ud og ind af cellekernen er blevet observeret på enkelt celleniveau og i cellepopulationsstudier efter ativering med sig-nale-molekylet TNF, men funktionen af disse oscillationer er stadig uklar. I dette projekt analyserer vi egenskaberne af oscilltionerne i NF-κB-aktivitet i cellekernen, som er genereret af det hurtige feedback fra inhibitorproteinet IκBα under det noget langsommere feedback fra A20 og A20-lignende proteiner. Vi finder, at denne struktur med to feedback-løkker inde i hinanden gør det muligt at ændre perioden af oscillationer meget specifikt. Derudover gør vi os overvejelser om, hvordan gener potentielt kan afkode den information, der ligger i oscillationerne.

Man ved, at inhibitorproteinet IκBα regulerer aktiviteten af NF-κB i cellekernen ved at eksporter den ud i cytoplasmaet. I et uafhængigt studie, hvor vi betragter interaktionen mellem NF-κB og DNAet, finder vi, at IκBα ikke kun eksporterer NF-κB ud af cellekernen, men at den aktivt fjerner NF-κB fra DNAet. Denne effekt har vist sig at være specielt vigtig ved tilstedeværelsen af store mængder uspecifikke DNA-bindingssteder, hvor IκBα er ansvarlig for hurtig NF-κB aktivitet og for at holde niveauet af NF-κB lavt i cellekernen, når cellen ikke bliver stimuleret.

Dernæst har vi set på interaktion mellem celler i et væv og på de mønstre, der dannes i cellernes proteiniveau i det tidlige udviklingsstadi. Vi har simulert væksten af væv på fire forskellige måder og testet deres modstand mod mutationer. Vi har fundet ud af at et væv er mere robust overfor mutationer, hvis cellerne er i en ordnet tilstand, og at mutationer har en større effekt på de omliggende celler, hvis den muterede celle ligger på kanten af et ustabil område. Effekten af mutationerne er større, når man muterer en aktiv celle til at være inaktiv, end når man muterer en inaktiv celle til at være aktiv.

Det fjerde projekt omhandler interaktionen mellem mennesker. Vi bruger netværksanalyse til at afkode højtilpassede interaktionsdata mellem modne og henholdsvis for tidligt fødte barn og born, som er født til tiden. Ved at sammenligne de to grupper finder vi, at interaktionen med de for tidligt fødte born ofte er mindre harmonisk. Vi finder, at mødrene til de for tidligt fødte born ofte befinder sig i to ikke-tilløbende adfærdsmønstre overfor barnet: De er enten påtrængende eller uengagerede. At sammensætte det meget følsomme barn med en påtrængende/uengageret mor kan gøre barnet mere modtageligt for følelsesmæssige, kognitive og adfærdsmæssige forstyrrelser senere i livet.
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Introductory Remarks

Life is dynamic. Changes in time can be observed on all scales, whether they concern the seasons of the year, our daily 24h rhythm or our need for chocolate. The driving motivation for many researchers is indeed how these fluctuations arise, how they are sustained and how they are dealt with. The complexity of biological organisms can, however, make it difficult to understand and interpret what we see.

Computational modeling of biological phenomena has over the past decades become an increasingly integrated tool for understanding and predicting biological responses. The interplay between mathematical models capable of capturing and processing complexity beyond most human minds, and the experimentally achieved insight of the biology provides a strong combination to reveal so far unknown features of the system. This idea of using computation to address biological questions has led to a variety of computational approaches, all under the common name of Systems Biology.

Systems biology can be divided into two main approaches; top-down and bottom-up. Top-down systems biology is data-driven, network structure models based on genome wide ‘omics’ data, with the overall aim of identification of molecular interaction networks on the basis of correlated molecular behavior observations. A top-down systems approach can provide some information on which proteins interact with which. The emergent properties of the system can be deducted from a model like this, but it can often be difficult to understand why those properties emerge as the model is complex and it can be hard to qualitatively distinguish between what is necessary for those properties and what is details. Bottom-up systems biology is on the other hand dominated by small scale mathematical simulations of a given pathway, looking at the mechanisms through which functional properties arise in the interaction of known components.

The primary focus of this thesis is small scale modeling with the aim of gaining dynamic insight into a specific part of a pathway. The main challenge when performing small scale modeling is first of all to isolate the system of interest from a complex organism and to get the right parameters. Small scale modeling is mainly performed on top of cell culture knock-out and knock-down experiments, which is the current way of getting the kind of time series data needed for the modeling. Despite the fact that these experimental observations can be far from reality, this type of modeling might still give us some insight to specific parts of the organism.

On every level, life is strongly dependent on interactions. Proteins interact and react, regulating the expression of other proteins. Without these interactions there will be no life in the cell. Cells on the other hand must always sense and signal to their surround-
ings -- whether it is a single cell organism or a cell in a body -- to get food, move away from toxins and to know the general state of the organism it is in. All cells together make an organism which again has, and to some extent is dependent on, the ability to interact with others of its kind. I have together with my co-workers used computational modeling to approach biological questions, each dealing with different kinds of interaction networks.

The first part of the thesis will introduce the concepts of mathematical modeling used in the presented work, and give a general introduction to cell-cell interaction networks and how human interaction networks can be interpreted. Chapter 3 focuses on the gene regulatory network of the transcription factor NF-κB. NF-κB is highly involved in many biological processes, and is the main player of our immune response. Our immune response depends on the processing of information about the current state of the body and of converting this into the required responses. This regulation is complex and much remains unknown. We have in this first study focused on the nuclear translocation of the transcription factor and how this is regulated by the inhibitor proteins IκBα and A20. Chapter 4 provides yet another step of detail in the regulation of the transcription factor, analyzing DNA-interaction and its disruption by the inhibitor protein IκBα.

In chapter 5 we leave the gene regulatory network and scale up to the dynamics of cell-cell interaction in tissue. A refined system of genes orchestrates the growth of tissues and organs during development, all of which will later span the complete organism. Cells in a tissue, through lateral inhibition, end up in different states making a fine-grained pattern across the tissue. We have looked into the formation of different patterns of cell states and how mutation in single cells in the tissue can affect the surrounding cells.

A basic instinct in humans and animals is to decode behavioral patterns to which we can adjust and tune our own behavior. We have in the project presented in chapter 6 looked at the specific interaction between mothers and infants, comparing infants born preterm with infants born full-term. Preterm infants are more likely to experience a developmental delay than full-term infants, but reported data on the behavior of the preterm infants are inconsistent. Here we aim to eliminate this inconsistency by an in-depth analysis of complex interaction networks and transition patterns in the combined behavior of the mother and the infant.

I hope to present my work in an interesting and inspiring fashion, reflecting the challenges I have met on the way but also the enthusiasm and the exciting paths I have been on.
Background

I will, in this first part of the thesis, introduce the general concepts used for the mathematical modeling presented in this thesis and give a brief introduction to the analysis of networks.

2.1 Gene Regulatory Networks

Gene regulatory networks are the type of networks I have studied in the first project and I will in the following give details of how we model transcription regulation and oscillations as part of network dynamics.

A cell is a crowded and complex environment through which proteins are constantly navigating to perform their individual roles for the cell to function and survive. The state of the cell is regulated by molecular interactions, starting from the membrane proteins sensing the environment outside of the cell and all the way down to the proteins controlling gene expression. Gene expression is one of the main mechanisms responsible for the state of the cell as it initiates the synthesis of new proteins. Gene expression itself can again be regulated on several levels by yet other proteins. The different genes and their products form a network where the nodes are the chemical species (protein, mRNA, DNA etc.) and the links are the type of interaction (binding, phosphorylation, degradation etc.). This kind of a network is called a gene regulatory network (GRN). See figure 2.1.1 for two examples of GRNs, the mammalian NF-κB and p53 stress response pathways, which are, respectively, important for the immune response and cell growth/death.

Modeling of GRNs can be done in multiple ways, e.g. as Boolean [Kauffman, 1969] or Bayesian [Friedman et al., 2000, Pearl, 1988] networks, but a common way is to use deterministic modeling, which usually uses coupled ordinary differential equations (ODEs). This approach is reliable as long as the size of the system is large enough for small fluctuations not to play a critical role in the dynamical behavior of the system. Stochastic modeling takes these fluctuations into account by adding noise to the system, e.g. by using the Gillespie algorithm or by Monte Carlo simulations. With the use of these kind of methods one can simulate the perturbations in the system when the number of molecules is less than 100 and stochastic interactions are essential for the dynamics.
Modeling Transcription Regulation

Gene expression is initiated by the presence and interaction of multiple transcription factors at the exact same time. This leads to RNA polymerase recruitment which, during the process of transcription, produces protein coding mRNA. Transcription is a dynamic event (figure 2.1.2) and the activity of the polymerase is proportional to the binding fraction of the transcription factor to the DNA site. This process is set by the dissociation constant of the protein-DNA complex.

The bound and un-bound fractions of the transcription factor-DNA complex can be derived both through mass action kinetics or by using statistical mechanics. Mass action kinetics is based on the on- and off-rates of the transcription factor binding to the DNA site whereas the statistical mechanics approach is based on the probability of the transcription factor binding to the DNA. I will in the following go through how we can derive the binding fraction in both ways, and how they lead to the same result.
First I will introduce a simple equation of the change in protein level (A) over time, given by
\[
\frac{dA}{dt} = c - \frac{\ln 2}{\tau} A
\]  \hspace{1cm} (2.1.1)

The change in protein level $A$ over time is a combination of the rate of production $c$ and the rate of protein degradation, which is inversely proportional to the half-life $\tau$ of the protein. This equation is commonly used in ODE based modeling which I will return to later in this chapter.

![Diagram of transcription: NFkB, koff, kon, operator site (O), Gene, Gene transcription.]

Figure 2.1.2: Transcription is a dynamic process and the expression of a gene is dependent on the relative fraction of the binding and un-binding of the transcription factor(P) to the operator site(O) of the gene.

The on- and off-rates of protein-DNA binding is poorly understood and many factors might combine to account for the overall dynamics of a transcription factor. The rate of protein binding to DNA is determined by factors such as diffusion, how easily the transcription factor finds the site, the affinity for the site and the concentration of DNA. A protein can perform a 1D search for the correct site by weak non-specific binding to the DNA it first encounters followed by sliding/moving along the DNA from the site of the initial binding. This can be combined with the more efficient 3D search where the protein is searching the DNA by diffusion. In the combined situation the protein does a local 1D search as well as jumping from one piece of DNA to the next, increasing the area of search. The off-rate is more complex and can be influenced by all kinds of other activity in the cell, such as local transport, small molecules, conformation change and thermal motion [Sneppen and Zocchi, 2005]. This will be further touched on in chapter 4 on page 35.

Mass Action Kinetics

Mass action kinetic modeling predicts features of a system of two or more reactants in dynamic equilibrium. Here we are looking at the complex formation of the transcription factor (P) and the DNA site(O):

\[
[P] + [O] \xrightleftharpoons{\text{koff}} \text{[PO]}
\]

where $k_{\text{on}}$ is the rate of complex formation and $k_{\text{off}}$ is the rate of complex breakdown, in this case the release of the transcription factor from the DNA site, see figure 2.1.2. The free amount of protein and DNA is given as the concentrations $[P]$ and $[O]$. The equilibrium is reached when the reaction rates in the two directions balance:

$$k_{\text{on}} [P] \cdot [O] = k_{\text{off}} [PO].$$
From this we can define the dissociation constant $K_D = \frac{k_{on}}{k_{off}}$ which allows us to formulate the amount of bound transcription factor as

$$[PO] = [O] \frac{[P]}{K_D}.$$  

We assume that the number of transcription factor ($P$) molecules are much larger than the number of DNA sites, $[P] \gg [O]$, as the number of sites can be as small as 1 compared to $10^{-100}$ transcription factor molecules. The amount of free transcription factor can under this assumption be estimated to be equal to the total amount of transcription factor, $[P_{\text{free}}] \sim [P_{\text{total}}]$. The amount of free DNA sites must, however, be corrected by how many of the total sites are already occupied.

$$[O_{\text{total}}] = [O_{\text{free}}] + [PO].$$

When combining the two expressions for the free amount of $P$ and $O$, we get an expression for the concentration of bound transcription factor, $P$;

$$[PO] = ([O_{\text{total}}] - [PO]) \frac{[P]}{K_D}$$

which by solving for the complex ($[PO]$) gives us

$$[PO] = [O_{\text{total}}] \frac{[P]}{K_D} \frac{1}{1 + [P] K_D^{-1}}. \quad (2.1.2)$$

We can in a similar way write the concentration of free DNA sites as,

$$[O_{\text{free}}] = [O_{\text{total}}] \frac{1}{1 + [P] K_D^{-1}}. \quad (2.1.3)$$

The relative probability of the on state with respect to the off state is given as

$$\frac{[PO]}{[O_{\text{free}}]} = \frac{P_{on}}{P_{off}} = \frac{[P]}{K_D}. \quad (2.1.4)$$

which we will use later.

The half-life of the $[PO]$ complex is given as

$$T_{\frac{1}{2}} = \frac{\ln(2)}{k_{\text{off}}}. $$

**Statistical Mechanics**

Finding the bound and un-bound fractions of protein to DNA through statistical mechanics is based on the binding probabilities. The probability of a protein ($P$) binding to an operator site ($O$) is given by the difference in Gibbs free energy between the bound and the unbound state, $\Delta G^\prime = G(\text{bound}) - G(\text{unbound})$. The state of the lowest free energy is the most probable state to be in, thus when $\Delta G^\prime < 0$ the bound state is more energetically favorable [Sneppen and Zocchi, 2005].

The statistical weight ($Z$) of a state is given by the number of ways the state can be realized, multiplied by the energy dependent probability of the state being realized, the
Boltzmann factor $e^{-\Delta G'/k_B T}$. The statistical weight of a protein being bound to a DNA site is given by

$$Z(\text{on}) = \frac{1}{(N-1)!} \left( \int_V \int \frac{d^3 r d^3 p}{h^3} e^{-p^2/(2 m k_B T)} \right)^{N-1} e^{-\Delta G'/k_B T},$$

where $N$ is the number of molecules, $T$ is the temperature, $k_B$ is the Boltzmann constant, $m$ is the mass of one protein/molecule and $\hbar$ is Planck’s constant. Planck’s constant was originally formulated as the probability constant between the energy of a photon and the frequency of its electromagnetic wave. By dividing by Planck’s constant we are taking the discreteness of the phase space imposed by quantum mechanics into account [Sneppen and Zocchi, 2005]. The integrals are taken over all positions $r$ and momenta $p$ of one molecule in the volume $V$. The statistical weight of having no proteins bound to the DNA site is in a similar fashion given as

$$Z(\text{off}) = \frac{1}{N!} \left( \int_V \int \frac{d^3 r d^3 p}{h^3} e^{-p^2/(2 m k_B T)} \right)^N$$

We can through integration of the above integral obtain the statistical weight of the state where one transcription factor is bound to the DNA site:

$$Z(\text{on}) = \left( \frac{V (2 m k_B T \pi / h^2)^{3/2} (N-1) \exp \left( \frac{-\Delta G'}{k_B T} \right)}{(N-1)!} \right)^{N-1}$$

where the volume $V$ results from the integral over the three spacial coordinates. This expression can be evaluated to

$$Z(\text{on}) \approx \left( \frac{c}{\rho} \right)^{N-1} \exp \left( \frac{-\Delta G'}{k_B T} \right)$$

where $c = \left( \frac{2 m k_B T \pi}{h^2} \right)^{3/2}$ and $\rho = \frac{N}{V}$ defines is the density of the free particles. In deriving the above expression we have used Sterling’s formula $N! \approx \left( \frac{N}{e} \right)^N$ and the approximation of $(N-1)/N \approx 1$. Similarly for the unbound state we find

$$Z(\text{off}) \approx \left( \frac{c}{\rho} \right)^N$$

The system of $N$ particles in a volume $V$ now has the combined statistical weight of $Z = Z(\text{on}) + Z(\text{off})$ with the probability of being in the on state $P_{\text{on}} = \frac{Z(\text{on})}{Z}$ and to be in the off state $P_{\text{off}} = \frac{Z(\text{off})}{Z}$. The relative probability of being in the on-state with respect to the off-state can then be written as

$$P_{\text{on}} = \frac{P_{\text{off}}}{c} \exp \left( \frac{-\Delta G'}{k_B T} \right).$$

When comparing this expression of the binding probability ratio with the one derived through mass action kinetics, equation 2.1.4 on the preceding page, acknowledging that the concentration of transcription factor $[P]$ is the same as the density $\rho$ of the transcription factor, we now have an expression of the relationship between the dissociation constant $K_D$ and the binding energy $\Delta G'$:

$$K_D = c \cdot e^{\frac{\Delta G'}{k_B T}}.$$
c is defined in equation 2.1.5 on the preceding page and is just like $K_D$ in units of concentration, but c is measured in units of particles per unit volume and $K_D$ is measured in moles per unit volume. The binding energy per molecule is given by $\Delta G'$ and if we now denote the binding energy per mole $\Delta G$, we get $K_D = |M| \cdot e^{-\frac{\Delta G}{k_B T}}$.

Continuing, we now standardize and use all statistical weights normalized in terms of $Z_{off}$. This gives the two expression for the on and off state as

$$Z_{on} = [P] \cdot \exp \left( -\frac{\Delta G'}{k_B T} \right) = \frac{[P]}{K_D} \quad \text{and} \quad Z_{off} = 1.$$  

From this we can calculate the fractions of the bound and unbound states, given by

$$\frac{P_{on}}{P_{on} + P_{off}} \quad \text{and} \quad \frac{P_{off}}{P_{on} + P_{off}},$$  

respectively. This can likewise be expressed by the statistical weights,

$$\frac{Z_{on}}{Z_{on} + Z_{off}} \quad \text{and} \quad \frac{Z_{off}}{Z_{on} + Z_{off}}.$$  

When substituting the normalized expression for the statistical weights we get

$$\frac{[P]}{K_D} + 1 \quad \text{and} \quad \frac{1}{1 + \frac{[P]}{K_D}}$$  

which are equivalent to the bound and unbound fractions found by the mass action approach earlier discussed, equation 2.1.2 and 2.1.3 on page 6.

By combining the expressions found for the bound and the unbound fractions with the simple equation for the change in protein level over time, equation 2.1.2, we get a more complete mathematical description of the change in protein level over time. This expression includes protein production as a function of transcription factor activity which is proportional to the concentration of transcription factor (P) • DNA complex in the case where the transcription factor activates the production of A;

$$\frac{dA}{dt} = [O_{tot}] \cdot \frac{[P]}{K_D} \cdot \frac{1}{1 + \frac{[P]}{K_D}} - \frac{1}{\tau} A \quad (2.1.6)$$  

Cooperative Binding

Regulatory effects often involve the binding of more than one molecule, and it has even been found that there is an enhancement of the binding to a macromolecule, such as DNA, when one molecule is already bound. This effect is called cooperative binding. Dimers can also bind cooperatively to a DNA site, as in the case of the phage $\lambda$ [S neppen and Zocchi, 2005], but two molecules binding to one DNA site as a dimer does not characterize as cooperative binding. The bound fraction of protein binding cooperatively to a DNA site can be expressed in the same fashion as above. We now have $n$ identical proteins (P) binding to the same promoter site (O),

$$nP + O \leftrightarrow P_n O$$
with $K_D = [P]^n [O]/[P_n O]$ and $[O_{total}] = [O_{free}] + [P_n O]$, which gives us a final expression of the concentration of the protein-DNA complex;

$$[PO] = [O_{total}] \frac{([P] / K_D)^h}{1 + ([P] / K_D)^h}$$

where $h$ is the Hill coefficient of the reaction and is a measure of the cooperativity. $h = 1$ is a non-cooperative reaction, $h > 1$ is a cooperative process and when $h < 1$ the affinity of the molecule binding to the DNA will decrease when another molecule is already bound. The Hill coefficient is given as the slope of the $\log([P_n O]/[O])$ vs $\log([P])$ plot. This will be used later when deriving the models of transcription factor activation of target genes in chapter 3.

I will in the following section give a brief conceptual overview of deterministic mathematical models of oscillations, focusing on a specific architectural feature of a gene regulatory network, namely feedback loops. Feedback loops are a commonly observed feature in genetic regulatory network and they will in the following be put in the context of modeling oscillatory control.

### Feedback Loops and Modeling Oscillatory Control


*Living cells continuously adjust gene expression patterns in response to changing environmental conditions. A simple way of encoding the presence of a stress or stimulus is to shift the concentration of a signaling molecule from one steady-state level to another. This scheme has potential disadvantages including the cost of continuous production of signaling molecules at a high level and unwanted cross-talk between pathways. It is not surprising, therefore, that cells often encode information about environmental changes in complex time-varying signals. This section deals with one subclass of such systems: those that exhibit oscillations.*

The most obvious examples of periodic behavior are circadian rhythms [Kitayama et al., 2008, Dong and Golden, 2008] and cell cycles [Lu and Cross, 2010]. Much more rapid oscillations are seen in the levels of cellular calcium [Zhu et al., 2008]. Many hormones also show intermittently periodic behavior and pulsatile secretion [Ciccone et al., 2010]. Figure 2.1.1 shows two such systems where oscillations have been observed: the NF-κB [Hoffmann et al., 2002, Nelson et al., 2004, Lee et al., 2009, Bartfeld et al., 2010] and the p53 [Geva-Zatorsky et al., 2006, Hamstra et al., 2006], which is important for the immune response and cell growth/death, respectively. Additionally, oscillations have been observed in the somitogenesis systems [Aulehla and Herrmann, 2004, Dequeant et al., 2006, Aulehla et al., 2008], which is important in embryo development.
Minimum Ingredients for Generating Oscillations

The minimum requirement for oscillations is a negative feedback loop with a time delay [Tiana et al., 2007]. A feedback loop is a closed cycle of nodes, representing genes, proteins, mRNA, etc. (henceforth: ‘regulators’), each affecting the concentration or activity of the next node through activation or inhibitory links (figure 2.1.3 (A)). A negative feedback loop is one with an odd number of inhibitory links. A small perturbation of one regulator will perturb the next one in the loop, which will increase or decrease the concentration of the next regulator, and so on, until the signal returns to the original regulator. The original perturbation will be canceled if there are an odd number of inhibitory links. Thus, negative feedback tends to produce homeostasis. The faster the signal goes around the loop, the quicker perturbations are nullified and therefore the tighter the homeostasis. However, if the signal goes around sufficiently slowly, i.e., with a distinct time delay, then this homeostasis can be broken. Negative feedback will still try to counteract perturbations, but the delay can make the regulator concentrations repeatedly overshoot their homeostatic levels and so oscillate (figure 2.1.3 (B)).

There are several ways to obtain an effective time delay [Tiana et al., 2007], some of which are listed below:

1. Processes that take a minimum amount of time. For example, transcription and translation, figure 2.1.3 (B.1). The simplest negative feedback loop is a single regulator inhibiting its own production/activity with an explicit time delay described by the equation

\[ \frac{dx}{dt} = \alpha/(K + x(t - \tau)^h) - \gamma x \]

As the time delay in this loop is increased, the behavior shifts from homeostasis to damped to sustained oscillations.

2. Many intermediate steps, i.e., a long feedback loop. Each step adds to the overall time delay, figure 2.1.3 (B.2).

3. Switch-like responses, where a regulator must reach a threshold concentration before it acts on the next in the loop, figure 2.1.3 (B.3).
4. **Saturated degradation**, where the degradation of a regulator is delayed by saturated complex-formation, figure 2.1.3 (B.4).

Figure 2.1.4 shows the core feedback loops generating oscillations in the NF-κB and p53 systems. The NF-κB model is taken from [Krishna et al., 2006] and the p53 model has been simplified from the models of [Hunziker et al., 2010] by assuming all complexes are in quasi-equilibrium. Both models use saturated degradation (terms in shaded boxes) to generate the sustained oscillations shown in the lower panels. The corresponding dynamical solutions are shown in the lower part of each panel, where the upper graphs show the protein activity of the transcription factor and its inhibitor protein; (A) $I/(0.05 + P)$, (B) $M * P / (0.05 + P)$, line colors matching protein colors in the schematics above. The times when the relevant complexes are saturated are indicated by the flat peaks of the blue curves (bottom panels) that plot: (A) $I/(0.035 + I)$, (B) $P / (2 + P)$, and the dotted lines show the associated inhibitors.

**Modeling Negative Feedback and Time Delay**

The model of Hes oscillations in [Jensen et al., 2003] is an example of case (1) above, where an explicit time delay in the production of Hes mRNA (which is inhibited by Hes protein) represents the amount of time taken for transcription, translation and then nuclear import of Hes protein (the identical model was published again in [Monk, 2003]). Models using explicit time delay have been widely used to model oscillations, for example, in respiration patterns and hematopoiesis [Mackey and Glass, 1977], p53 [Tiana et al., 2002], somitogenesis [Lewis, 2003], insulin secretion [Li et al., 2006], and the hypothalamic-pituitary-adrenal axis [Walker et al., 2010]. Using explicit delays is somewhat ad-hoc and we find it more satisfying to model the specific molecular processes that produce delay.

The conceptually simplest way to do this is case (2) above. For example, an oscillator formed by a loop of six nodes, involving three proteins, and their mRNA, each protein inhibiting transcription of the next (figure 2.1.3 (B.2)), has been modeled and experimentally realized in ref. [Elowitz and Leibler, 2000]. Another synthetic circuit that oscillates due to many intermediate steps was constructed in [Stricker et al., 2008]. More complex is case (3) where one regulator affects the next only when it reaches a threshold concentration. The delay arises from the time taken for the threshold to be reached. Simple models of oscillations have implemented this by using either a highly cooperative interaction with a large ($\geq 8$) Hill coefficient [Goodwin, 1965] or positive feedback loops [Tsai et al., 2008, Krishna et al., 2009]. Both large Hill coefficients and positive feedback result in sigmoidal, switch-like responses and hence a threshold concentration below which the response is essentially zero. Combining positive and negative feedback has the advantage of making oscillations more robust and yet tunable [Tsai et al., 2008, Krishna et al., 2009], and has been used to model many phenomena, including cell cycles [Pomerening et al., 2005, Gerard and Goldbeter, 2009], circadian rhythms [Dong and Golden, 2008, Ferrell et al., 2009, Cookson et al., 2009], division site localization in E. coli [Howard and Kruse, 2005], and p53 oscillations [Gilberto et al., 2005, Geva-Zatorsky et al., 2006, Puszyński et al., 2008], as well as to design synthetic oscillators [Atkinson et al., 2003, Fung et al., 2005, Stricker et al., 2008].

These time-delay mechanisms are, however, not mutually exclusive, and systems typically use several of these mechanisms, each contributing to the overall delay.
Figure 2.1.4: Modeling negative feedback and time delay. Core feedback loops generating oscillations in the NF-κB and p53 systems. Rectangles denote mRNA and rounded rectangles represent proteins. Also shown are equations of three-variable models for each system. Capital letters signify protein/mRNA concentrations, Greek letters are degradation rates, subscripted \( k \)'s denote maximal production rates, and subscripted \( K \)'s are dissociation constants. Parameter values used were: (A) \( k_t = 5.4 \text{min}^{-1}, N_{\text{tot}} = 2.0 \mu M, K_I = 0.035 \mu M, K_N = 0.029 \mu M, k_c = 1.0 \mu M^{-1} \text{min}^{-1}, k_I = 0.25 \text{min}^{-1}, \delta = 0.018 \text{min}^{-1}, \beta = 0.017 \text{min}^{-1}, \alpha_{\text{IKK}} = 1.0 \text{min}^{-1} \); (B) \( k_s = 1000 \mu M \text{min}^{-1}, K = 2 \mu M, k_c = 0.03 \mu M^{-1} \text{min}^{-1}, k_t = 1.4 \text{min}^{-1}, \delta = 2 \text{min}^{-1}, \beta = 0.6 \text{min}^{-1}, \alpha = 1 \text{min}^{-1} \).

The NF-κB Signalling Pathway

NF-κB, a main player in our immune response, regulates the expression of hundreds of genes and is implicated in a large number of diseases, including cancer, heart diseases and asthma [Pahl, 1999]. Nuclear translocation of NF-κB, necessary for its transcription factor 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 [Nelson et al., 2004, Lee et al., 2009].

NF-κB is regulated by, among others, the inhibitor proteins IκB which bind the nuclear transcription factor and export it out into the cytoplasm. Stimulation by TNF activates the IκB kinase (IKK) which in turn causes the phosphorylation, and subse-
quent degradation, of the IκB inhibitor proteins, thus releasing NF-κB. Free NF-κB translocates to the nucleus inducing transcription of hundreds of genes, some of which code for the inhibitor proteins, IκBε and IκBo, and A20-like proteins. In turn, the IκB proteins inhibit the NF-κB transcription factor by actively exporting it out of the nucleus.

Model of NF-κB Signaling

I will in the next section go more into detail about saturated degradation (case (4)), which drives oscillations in the NF-κB system. Oscillations in p53 are also caused by saturated degradation but I will not touch further upon this here.

The negative feedback loop underlying oscillations in NF-κB has two legs (figure 2.1.4 (A)): (i) NF-κB activates IκBo production, (ii) IκBα inhibits NF-κB by sequestering it in the cytoplasm. Leg (i) is active when there is little IκBα, so most NF-κB is free to enter the nucleus, causing IκBα levels to rise. Free NF-κB levels then fall rapidly as it gets bound to newly synthesized IκBα. In the model of figure 2.1.4 (A) [Krishna et al., 2006], the amount of NF-κB-IκBα complex has a Michaelis-Menten form: \( NcI / (KI + I) \), where \( I \) is the IκBα concentration and \( Nc \) is the total cytoplasmic NF-κB concentration. The binding is strong, i.e. \( K_I \) is small, so IκBα levels quickly become large enough to saturate NF-κB, at which point the amount of complex becomes equal to \( Nc \) and independent of \( I \). This is leg (ii) of the feedback. Now there is no further production of IκBα, so its levels will eventually fall. However, only IκBα molecules that are bound to NF-κB are subject to IKK-dependent degradation so the degradation rate (second term in the \( dI / dt \) equation in figure 2.1.4 (A)) depends not on the amount of IκBα present, but on the level of the complex. Because the complex level is saturated and equal to \( Nc \), most of the time (see blue curve in figure 2.1.4 (A), bottom panel) we call this ‘saturated degradation’. If, instead, IKK action led to degradation of both free and complexed IκBα, then the degradation rate would be proportional to \( I \), which would make \( I \) decrease exponentially fast. In contrast, with saturated degradation \( I \) decreases relatively slowly when it is large, because only the small amount of IκBα complexed with NF-κB is sensitive to degradation, resulting in the more rounded shape of \( I \) vs. time seen in figure 2.1.4 (A, yellow curve). This provides a sufficient time delay to generate oscillations.

NF-κB is not only regulated by the inhibitor proteins of the IκB family acting directly on the transcription factor. Proteins like A20 also play a regulatory role by acting up-stream in the pathway.

The A20-like proteins are important regulators of late IKK activity and has experimentally been shown required for the drop in NF-κB activity separating early and late phase response to TNF when measured in bulk [Werner et al., 2008]. The A20 family of deubiquitinating enzymes includes A20, CYLD, Cezanne and others. Cells deficient in A20 like proteins show persistent IKK activity and elevated late phase NF-κB activity and develop severe inflammation and cachexia [Lipniacki et al., 2004, Lee et al., 2000, Trompouki et al., 2003, Brummelkamp et al., 2003, Kovalenko et al., 2003, Enesa et al., 2008]. In the following I will give a brief introduction to the pathway lying above IKK activation and nuclear translocation of NF-κB.

The Up-Stream Pathway

A full understanding of the inflammatory pathway of TNF triggered NF-κB activity in the nucleus still remains to be revealed. The overall dynamics of the transcription
factor is well understood together with the very first protein assembly taking place at the membrane when TNF activates the cell. The link between the membrane located protein assembly and the release of NF-κB into the nucleus is however still hazy. The apparent connection, the ubiquitination of the Nuclear factor κB Essential Modulator (NEMO), still has many interpretations and its activation remains mostly unknown.

Figure 2.1.5: Schematics of the up-stream part of the NF-κB pathway. Proteins are round and squared and adapter proteins are marked with names and no symbols. After IKK activation and subsequent degradation of the inhibitor protein, IκB, NF-κB is released to enter the nucleus where gene expression is initiated.

First I will state the previous view of the activation of the pathway leading to the nuclear translocation of NF-κB: The pathway is activated by TNF interaction with the TNF receptor and subsequent receptor trimerization. Receptor interacting protein 1 (RIP1) is recruited to the TNFR1 receptor by the adaptor molecule TRADD and is believed to be ubiquitinated by a chain of lysine 63 linked ubiquitin molecules (K63 linked ubiquitin chain) [Newton et al., 2008] with help from the E3 ligase TRAF5. The K63 linked ubiquitin chain is not labeling the protein for degradation by the 26S proteasome as other ubiquitin chains do, but functions as a scaffold for proteins with ubiquitin binding sites. The K63 linked ubiquitin recruits TAB2 to the complex of TRADD, TRAF2/5 and RIP, where it functions as an adapter for TAK1 and NEMO. Once bound to the K63 ubiquitin chain, NEMO forms a complex with IKKα and IKKβ and the whole IKK complex is activated by the phosphorylation of IKKβ by TAK1. The IKK complex is now able to phosphorylate the NF-κB inhibitor protein, IκB, targeting it for degradation by the 26S proteasome which allows NF-κB to translocate to the nucleus, figure 2.1.5. This represents the previous view on how the signal propagates down the pathway activating nuclear translocation of NF-κB.

This view of TNF induced NFκB activation has evolved and been prominent the past years but lately new research has shed light on an alternative or additional factor in the NF-κB activation.

Linear Ubiquitin Chain Assembly Complex (LUBAC) is a protein complex consist-
ing of the two ring finger proteins HOIL-1 and HOIP [Lo et al., 2009]. LUBAC functions as a ligase complex with the full function of conjugation linear ubiquitin chains to substrates. LUBAC has been shown to activate the NF-κB pathway by attracting linear ubiquitin chains to the CC2-LZ domain of NEMO [Lo et al., 2009] in an Ubc13 independent manner and knockout studies of the two LUBAC proteins show suppressed NF-κB activity.

NEMO has a higher affinity for interaction with linear ubiquitin chains than with K63 linked chains [Lo et al., 2009, Rahighi et al., 2009]. The domain at which the linear di-ubiquitin chain interacts with NEMO is called the CoZi domain [Rahighi et al., 2009] and by structure analysis it has been described how the two linear ubiquitin molecules fit into the site on NEMO and how it takes a chain of 4 or more K63 linked ubiquitin to achieve the same kind of binding as with linear ubiquitin [Rahighi et al., 2009]. This reveals how large amounts of K63 linked ubiquitin could seem to be associated with NEMO whereas the actual effective interaction is by linear di-ubiquitin molecules.

Additionally it has been shown that the TNF induced NF-κB activity is triggered independent of the K63 linked ubiquitin chain ligase, Ubc13 [Yamamoto et al., 2006]. This indicates that K63 might not play as dominant a role in the activation as first expected.

Based on the latest result there might be yet another way of activating the NF-κB pathway. It was shown how TRAF2, RIP1 and TAK1 are essential in the activating the pathway and these proteins interact with K63 linked ubiquitin for activation. However, the E2 ligase for K63 linked ubiquitin formation, Ubc13, has in turn been shown unnecessary for TNF induced NF-κB activation [Yamamoto et al., 2006].

These facts about the NF-κB pathway will be used in future chapters where we study models of NF-κB activity and the role of up-stream regulation of the transcription factor activity.
CHAPTER 2. BACKGROUND

2.2 Cell-Cell Interaction Networks

After having introduced gene regulatory networks in individual cells, I will now zoom out and imagine a group of cells, all having their own gene transcription network inside. Cells are constantly signaling to each other and sensing molecules from other cells nearby and far away. This enables cells to communicate to get news of the current state of the organism it is in, and act accordingly. For a single cell organism life may in some ways be more simple as it only has to take care of itself - but also more dangerous. A single cell organism such as *E. coli* is always looking for food and trying to avoid potential dangers like toxins, which it senses through receptor proteins on its surface and responds to by chemotaxing [Barkai and Leibler, 1998]. Cell-to-cell communication between bacteria happen through quorum sensing where the cell is monitoring the cell density and diversity of the bacterial population [Henke and Bassler, 2004]. We will in the following focus on cell-to-cell interaction in multicellular organisms.

Communication Between Neighboring Cells

The interaction between two cells can either be bi-directed or have only one direction. *Bi-directed* interaction can among other mechanisms happen through gap junctions where two cells are directly connected allowing molecules to pass freely between the cells. Another kind of bi-directed cell-cell interaction is through Delta-Notch [Sprinzak et al., 2010] where the two transmembrane proteins inhibit the activity of each other through direct membrane protein contact (see further details below). Cells can additionally affect each other at a distance using secreted molecules that travel by diffusion. The target cell might in this case be far away from the sending cell, and might not be signaling at the same time. This type of interaction is mainly *directed*, figure 2.2.2 on the facing page (A). We can, however, imagine the target cell upon stimulus from the sending cell will secrete molecules which can lead to a delayed *bi-directional* activation of the original cell.

Delta-Notch

Coordinated gene action directing the fate of individual cells during development results in defined, multicellular structures building up an organism. This action is an in-
terplay between many factors such as cell growth, migration, proliferation, differentiation, and death [Artavanis-Tsakonas et al., 1999]. Tissues are formed through repeated cell division, where neighboring cells enter different developmental pathways, resulting in a fine-grained pattern of different cell states [Greenwald, 1992]. The most common mechanism generating such patterns is lateral inhibition through Delta-Notch coupling [Sprinzak et al., 2010].

The two transmembrane proteins, Notch and Delta, repress each other. Delta is known to have two activities; it transactivates Notch in neighboring cells and cis-inhibits Notch in its own cell. The activation of Notch leads to nuclear translocation of the intracellular domain of Notch which lead to target genes’ expression. Genes up-regulated by Notch in the target cell inhibit the function of Delta and thus lead to repression of Notch activation in the reference cell, figure 2.2.2 (B). This dynamics generates a very sensitive switch between the two signaling states of sending (high Delta/low Notch) and receiving (low Delta/high Notch), which on the multicellular level can amplify small differences between neighboring cells and can result in patterns in cell state [Sprinzak et al., 2010].

Figure 2.2.2 (B) shows a schematics of the inhibition between Delta (DII) and Notch. The two different activities of Delta are given as transactivation, marked as a bridge (\(\wedge\wedge\)), and cis-inhibition, where Delta is drawn on top of Notch at the cell membrane. Activated Notch enters the nucleus there it up-regulates genes (blue rectangle), leading to repression of the activity of Delta. Figure 2.2.2 (C) shows an example of tissue patterns formation through lateral inhibition.

Tissue Growth

Cells are, during development, arranged in refined three-dimensional structures of organs of complicated shapes. Tissue can, however, be approximated as a 2 dimensional layer of cells arranged on an hexagonal lattice, as in figure 2.2.1 (C).

Tissue growth can happen in various different ways. Two main types are cell division and cell migration, figure 2.2.3. New cells added to the tissue will be in the same state as the mother cell, when the the cell is dividing, and the new cell is located right next
CHAPTER 2. BACKGROUND

Figure 2.2.3: Tissue growth through A: cell division and B: cell migration.

to the mother cell. If the division is taking place within the tissue new cells would be pushing the existing cells in the direction of division. The hexagonal lattice structure has 6 directions of orientation.

If a tissue is built by cell migration, cells from other parts of the developing organism are coming to join the existing group of cells. The new cells will be in a silenced state, figure 2.2.3 (B). We can additionally imagine lateral growth, where the top layer of cells all divide, expanding the tissue in only one direction.

Modeling Interaction

When modeling an interaction network between cells we use the same type of equations presented in the previous section for protein activity, equation 2.1.1 on page 5. The protein now represents the Notch protein in the cell, and the interaction between the cells is added as an extra term to the expression in eq. 2.1.1. The cells are arranged on a hexagonal lattice as seen in figure 2.2.2 (B), so that all cells have 6 neighbors which the cell is interacting with through repression. We use standard Michaelis-Menten kinetics of protein interaction and model the repression:

\[
\cdots \frac{1}{1 + \left(\frac{\text{cell} \, 1}{K_D}\right)^h} \cdots
\]

where \text{cell} 1 is one of the neighboring cells, \(K_D\) is the dissociation constant of the binding complex and \(h\) is the Hill coefficient. The repression from the neighboring cells can be either additive where the total effect is the sum of each component (an ‘or gate’), or it can be multiplicative which allows for synergistic effects of the combined repression (an ‘and gate’). Additive repression is now given as

\[
\cdots \frac{1}{1 + \left(\frac{\text{cell} \, 1}{K_D}\right)^h} + \frac{1}{1 + \left(\frac{\text{cell} \, 2}{K_D}\right)^h} + \frac{1}{1 + \left(\frac{\text{cell} \, 3}{K_D}\right)^h} \cdots
\]
expanded for all 6 neighboring cells. If the activity of one of the surrounding cells is zero or very low this cell will contribute with a '1' to the sum, and the protein activity of the reference cell will grow. A cell of high activity will result in a contribution of a number in the interval [0,1] leading to lesser increase in protein level of the reference cell. The maximal activity of the reference cell is in this way achieved when the cell is surrounded by low activity cells.

Multiplicative interaction holds some of the same dynamical aspects as the additive repression, but a main difference is that the terms are now multiplied together, canceling out contributions from zero activity cells. Please note that in this type of equation setup, one silenced cell is not completely diminishing the effect of the other active cells, as the cell protein levels are not directly multiplied. The protein levels contribute in the repressive form of \(1/(1 + \text{cell activity})\) which turns zero activity of one cell into an effective contribution of '1'. Contributions of < 1 from high activity cells will on the other hand bring down the protein level of the reference cell. Substantially higher total protein levels are reached through additive rather than multiplicative repression. The corresponding expression for multiplicative repression is given as

\[
\frac{1}{1 + \left(\frac{\text{cell } 1}{K_D}\right)^h} \cdot \frac{1}{1 + \left(\frac{\text{cell } 2}{K_D}\right)^h} \cdot \frac{1}{1 + \left(\frac{\text{cell } 3}{K_D}\right)^h} \cdots
\]

More details of the modeling will be provided in chapter 5.
2.3 Human Interactions Networks

A basic instinct in humans and animals is to decode behavioral patterns under which we can adjust and tune our own behavior. Understanding and predicting human behavior has thus always been of great interest. Recently, interest turned to quantitative analysis of human activities using mathematical models and network tools, addressing temporal and structural features of human communication [Barabasi, 2005, Borgatti et al., 2009].

Social sciences have used network analysis since the late 1950s, seeing societies as a "pattern of network of relationships obtained between actors in their capacity of playing roles relative to each other" [Nadel, 1957]. Equally early network analysis has been used to analyze the roles between husband and wife in urban British families, finding that the more socially connected the family was, the more they tended to stick to the traditional pattern of husband and wife roles [Bott, 1957]. These kind of studies use tools from the physical interpretations of networks.

Network Topology Analysis

A wide variety of systems can be described as complex weblike structures. One example is the cell which is best described as a complex network of genes and proteins connected by molecular reactions, as described above. Other examples of networks are the Internet as a complex network of routers and computers or social networks where nodes are human beings and links represent various social relationships spreading ideas and information. These kinds of systems have, especially in the last decade, motivated scientists to investigate the mechanisms that determine the topology of complex networks.

The early analysis of complex networks was dominated by graph theory, initially focusing on regular graphs but since the 1950s as random graphs. The first random networks were studied by Paul Erdős and Alfréd Rényi where we start with N nodes and connect every pair of nodes with probability p, creating a graph with approximately \( pN(N-1)/2 \) links distributed at random, [Erdős and Rényi, 1959, Albert and Barabási, 2002].

Lately, the question has for many scientists been whether the networks behind complex systems, such as the cell or social interactions, are fundamentally random. Intuitively we would say that there are some organization principles which would make them diverge from random, but the question is how to capture this. Below I will introduce some proposed concepts of this measure.

Small Worlds

Despite the often large size of a network there is in most networks a relative short path between any two nodes. This is the first property defining the small-world concept. The distance between two nodes is given by the number of links connecting them through the shortest path. A popular example of this is the 'six degrees of separation', a concept proposed by the social psychologist Stanley Milgram (1967) that almost every two people in the United States are connected by a path of acquaintances with a typical length of six. The average shortest path in a small-world grows as \( \log(N) \), where \( N \) is the number of nodes in the network.

A second property of small-world networks is a high clustering coefficient. Clustering is the formation of cliques in social networks, where all nodes in a groups are
connected to each other. The clustering coefficient is calculated for every node respectively and the clustering coefficient of a whole network is the average of all individual clustering coefficients, $C$. $C$ is given by

$$C = \frac{2E_i}{k_i(k_i - 1)},$$

where a node $i$ in the network has $k_i$ links connected to $k_i$ other nodes. The clustering coefficient of a random network is $C = p$, the node pairing probability.

Random graphs like the Erdős Rényi have the average shortest path which grows as $\log(N)$ but not a high clustering coefficient as the links are added with the same likelihood. Regular graphs have a high clustering coefficient but the average shortest path does not scale with $\log(N)$. Both are therefore not small-world.

Interestingly, [Watts and Strogatz, 1998] showed in 1998 that a regular graph with a very small number of additional random links have both properties of the small-world.

Broad Degree Distribution

The degree of a given node is the number of links going to and from the given node, and it will not be the same for all nodes in the network. The distribution function $P(k)$ is the probability that a randomly selected node has exactly $k$ links, and thus quantifies the variation in node degrees. As the links in a Erdős Rényi random network are placed randomly, a large fraction of the nodes will have approximately the same degree, which is close to the average degree $(\langle k \rangle)$ of the network. The degree distribution of a Erdős Rényi random network is a Poisson distribution with a peak at $P(\langle k \rangle)$. Most large networks do, however, diverge significantly from a Poisson distribution, and the degree distribution of these networks, such as the World Wide Web [Barabási and Albert, 1999], the Internet [Faloutsos et al., 1999] and metabolic networks [Jeong et al., 2000], has a power-law tail

$$P(k) \propto 1/k^\gamma.$$

These are referred to as scale-free networks as the change in behavior is independent of scale:

$$\frac{P(ak)}{P(k)} = \frac{P(a)}{P(1)}$$

as every time we multiply $P$ with $a$, the frequency $P$ is equally decreased by $1/\alpha$ [Sneppen and Zocchi, 2005, Albert and Barabási, 2002].

Preferential Attachment

The connection between two nodes was in the Erdős Rényi graphs assumed to be equally likely. It is, however, in real networks more likely to connect to already well connected nodes. This is called preferential attachment, and a way of achieving a scale-free network. An example of this is the tendency for a new manuscript to cite already well-known papers in the field.

This general background on networks sets the foundation for the project on human mother-infant interactions, presented in chapter 6.
Oscillations in Nuclear NF-κB Activity

A cell must constantly relate and respond to the surrounding environment through gene expression and other regulatory mechanisms. This regulation often relies on clusters of multiple interconnected feedback loops [Brandman et al., 2005, Trusina et al., 2008, Tsai et al., 2008], which in some cases makes the response more robust. For example: clustered negative feedbacks allowing for a robust adaptation [Ma et al., 2009] or interlinked fast and slow positive feedbacks adding to the robustness of a switch [Brandman et al., 2005]. In other cases the combined effect of several feedback loops is able to better regulate and tune a systems response [Justman et al., 2009]. In *Bacillus subtilis* two positive feedback loops provide regulation and robustness to the gene expression [Rao et al., 2004] and adaptive response otherwise mainly found in negative feedback circuits [Ray and Igoshin, 2010].

In paper 3 we investigate the case where feedback loops are arranged such that they are acting on different levels: one group acting directly on the transcription factor and one group acting up-stream. We refer to this type of topology as ’nested’ feedback loops, see fig. 3.2.1 (A). This configuration is inspired by the NF-κB regulatory system, introduced on page 12. NF-κB has several feedback loops but we are here focusing on the central feedback from IκBα, driving the oscillations in nuclear NF-κB activity, situated within the feedback of the A20-like proteins acting up-stream of the IκB kinase (IKK). The role of feedback loops in gene regulation has previously been discussed on page 9.

The following chapter is based on Paper 3: Benedicte Mengel, Sandeep Krishna, Mogens H. Jensen and Ala Trusina, Nested Feedback Loops in Gene Regulation. Under review.

3.1 Introduction

The physiological importance of the NF-κB transcription factor and its intriguing dynamical behavior has made it a center of attention for decades both from an experimental and theoretical point of view [Scott et al., 1993, Huxford et al., 1998]. The first computational model of the NF-κB pathway was proposed in [Hoffmann et al., 2002] 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., 2004] to analyze oscillations in single cells. Later, [Krishna et al., 2006] showed that the model can be significantly reduced while still capturing the essential dynamical features, in particular showing spiky oscillations in single cells.

To achieve high quantitative agreement with bulk data it is often required to have a detailed model, as presented by [Werner et al., 2008]. Here they have included \( \text{I} \beta \text{B} \), \( \text{I} \beta \text{B} \epsilon \) and \( \text{A}20 \) feedback loops in their previous model [Hoffmann et al., 2002]. Using this model they show that \( \text{A}20 \) lowers the resting level of nuclear NF-\( \kappa \text{B} \) activity, thereby providing control of the late phase of the TNF induced NF-\( \kappa \text{B} \) response. They additionally show that constitutive expression of \( \text{A}20 \) alone is sufficient for this effect.

Other studies have focused on details of where and how \( \text{A}20 \)-like proteins act in the pathway [Ashall et al., 2009]. To address the discrepancy in the nuclear activity of NF-\( \kappa \text{B} \), between bulk and single cell data [Kim et al., 2009, Ashall et al., 2009] introduced stochasticity and showed that averaging single cell stochastic dynamics leads to a smooth damped response in bulk. Additionally, [Hayot and Jayaprakash, 2006] have showed how fluctuations in the level of IKK can produce cell to cell variation in the NF-\( \kappa \text{B} \) period and amplitude leading to a damped bulk response.

The role of \( \text{A}20 \)-like proteins has, however, not been investigated in single cells where the nuclear response of NF-\( \kappa \text{B} \) is oscillatory [Nelson et al., 2004, Ashall et al., 2009]. Using the proposed model we aim to investigate the role of \( \text{A}20 \)-like proteins as modifiers of the NF-\( \kappa \text{B} \) oscillatory behavior in single cells.

### 3.2 Results

**Modeling NF-\( \kappa \text{B} \) Nuclear Response**

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**Figure 3.2.1:** Schematic drawing of the nested NF-\( \kappa \text{B} \) model  
A) Details of the NF-\( \kappa \text{B} \) pathway: TNF activates \( \text{I} \beta \text{B} \) kinase (IKK) which in turn causes the phosphorylation and subsequent degradation of the \( \text{I} \beta \text{B} \) inhibitor proteins, thus releasing NF-\( \kappa \text{B} \). Free NF-\( \kappa \text{B} \) translocates to the nucleus inducing transcription of the inhibitor proteins, \( \text{I} \beta \text{B} \epsilon \) and \( \text{A}20 \)-like proteins. The \( \text{I} \beta \text{B} \) proteins inhibit the NF-\( \kappa \text{B} \) transcription factor by actively exporting it out of the nucleus. \( \text{A}20 \)-like proteins act up-stream by inactivating IKK. Details of the IKK regulation module can be found in the insert box.  
B) Model response, top: NF-\( \kappa \text{B} \) nuclear/total ratio, bottom: IKK active/total ratio.

Here we propose a simple model extending the one presented by [Krishna et al., 2006] to include the up-stream feedback from \( \text{A}20 \). We focus on the nested feedbacks of the NF-\( \kappa \text{B} \) induced proteins in single cells and having a simple model allows for more
direct understanding of the dynamics. Figure 3.2.1 (A) shows a schematic representation of the model, consisting of two negative feedback loops centered around NF-κB: IκBα and A20-like proteins. I will henceforth denote the up-stream feedback ‘A20’, but it should be seen as a group of A20-like proteins all functioning at this level of regulation, including A20, CYLD [Jono et al., 2004] and Cezanne [Evans et al., 2001, Enesa et al., 2008].

IκBε has, from bulk experiments, been shown to dampen the oscillatory nuclear NF-κB activity driven by the fast feedback from IκBα [Kearns et al., 2007]. This dampening effect has however not been seen on a single cell level where the feedback from IκBε is thought to de-synchronize the oscillation resulting in an overall damped nuclear NF-κB response [Ashall et al., 2009]. Both in bulk and single cell experiments the late onset of IκBε transcription is seen, which was reported by [Kearns et al., 2007] and proven by [Ashall et al., 2009] to be due to the later assembly of the transcription machinery and thus the onset of the RNA polymerase, thus delaying IκBε mRNA by 37 min relative to the immediate synthesis of IκBα mRNA. We are here only modeling a single cell and have chosen not to include the effect of IκBε de-synchronizing as this is only relevant for larger cell population models. If IκBε is included in the model, the delay and the slower protein degradation will result in a weak dampening of the nuclear NF-κB activity.

We have made a deterministic model of a single cell treated with an incoming stimulus, TNF. We omit both the negative feedback from IκBε and other inhibitor proteins and only focus on IκBα driving the oscillations. Our model thus remains within the spirit of the earlier minimal model, [Krishna et al., 2006].

The Inhibitor Kinase, IKK

IκB Kinase (IKK) is the driving force of the system as its activation leads to the degradation of the IκB inhibitor proteins, 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, as discussed on page 13. We have here chosen to use the mechanism earlier proposed by [Ashall et al., 2009], modeling IKK activation as a three step process. IKK is converted from its neutral state to being active by the triggering signal, TNF, see figure 3.2.1 (A, insert box). Active IKK can turn itself off and go back into the neutral state before being activated again by the TNF signal. IKK is shut down by A20 which inhibits the transformation from inactive to neutral IKK thereby leaving IKK in an inactive state, see figure 3.2.1 (A).

The first 30 minutes of the IKK adaptation-like temporal profile in response to TNF stimulation is fitted such that it follows the experimentally observed IKK peak at about 15 minutes which then decreases to a new steady state after 30 minutes of TNF induction. This first part of the profile appears to be independent of A20 regulation [Werner et al., 2008]. The new steady state level is, however, determined by A20: it is high in the absence of A20 [Werner et al., 2008] and decreases with increasing concentrations of A20.

A20 protein affects only the late phase of the IKK temporal profile: the A20 mRNA level reaches maximum at 30 min and the effect of the protein is seen only after 45-60 min [Werner et al., 2005, Werner et al., 2008]. This feature is reproduced by the model where the level of IKK in the late phase is pushed 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. This feedback is slow, as IKK must first activate NF-κB leading to the production of A20 that in turn
shuts down the pathway through IKK.

Most rates and timescales used for conducting the model are, if possible, from the
existing literature. We have additionally adjusted rates manually 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
   90-120 min
2. Mutants with IκBα alone show enhanced oscillations
3. NF-κB resting level is lowered by A20

Model Description

The model is based on the three-variable model presented by [Krishna et al., 2006]
which we have extended to include A20 induction and feedback.

We use the following abbreviations:

\( N_n \) free nuclear NF-κB
\( I_m \) IκB mRNA
\( I \) free IκB
\( A20 \) A20 protein
\( IKK \) IκB kinase
\( IKK_i \) inactive form of IKK

The system of equations for the model are given below:

\[
\frac{dN_n}{dt} = k_N \frac{(1 - N_n)}{(1 + I)} - B(I_\alpha) \frac{N_n}{(\delta + N_n)}
\]  

\[
\frac{dI_m}{dt} = p + t \frac{N_n^2}{(1 + N_n^2)} - \gamma_m I_m
\]  

\[
\frac{dI}{dt} = I_m - \alpha_\alpha IKK \frac{(1 - N_n)}{1 + I} - \gamma I
\]  

\[
\frac{dA20_m}{dt} = \gamma_m A20_m - \gamma A20 A20_m
\]  

\[
\frac{dIKK}{dt} = T(1 - IKK - IKK_i) - \mu IKK^2
\]  

\[
\frac{dIKK_i}{dt} = \mu IKK^2 - \beta \frac{IKK_i}{\sigma A20^2 + 1}
\]  

where \( k_N \) is the rate of import of NF-κB into the nucleus, \( t \)'s are transcription rates
and \( \gamma \)'s are translation rates, \( N_{tot} \) is the total NF-κB concentration, \( K_\alpha \) is a ratio
between IκBα - NF-κB complex dissociation and formation rates, \( B \) determines the rate
of export of nuclear NF-κB, \( \delta \) is the concentration at which half of IκBα is bound in
complex with NF-κB, \( K_D \) is a dissociation constant and \( p \) is the NF-κB independent
transcription rate of IκBα. Further explanation of the parameters and rates can be
### RESULTS

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<th>Process</th>
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<th>Reference</th>
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</thead>
<tbody>
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<td>NF-κB nuclear import</td>
<td>$k_N$</td>
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<td>[Krishna et al., 2006]</td>
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<td>NF-κB nuclear export by IκB</td>
<td>B</td>
<td>$2.4 \cdot 10^{-4} \text{ min}^{-1}$</td>
<td>[Krishna et al., 2006]</td>
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<td>Conc at half IκB bound to NF-κB</td>
<td>$\delta$</td>
<td>0.0414</td>
<td>[Krishna et al., 2006]</td>
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<tr>
<td>$\alpha$</td>
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<td>[Krishna et al., 2006]</td>
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<td>$\alpha$</td>
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<td>$\gamma$</td>
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</tr>
<tr>
<td>$\gamma$</td>
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<td>→ IκB0 mRNA (NF-κB )</td>
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<td>[Krishna et al., 2006]</td>
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<td>IκB0 → deg</td>
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<td>Fitted, period of 90 min</td>
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<th>Process</th>
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<td>A20 mRNA</td>
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<td>[Werner et al., 2008]</td>
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<td>Assumed same as for IκB</td>
</tr>
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<td>[Werner et al., 2008]</td>
</tr>
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<td>A20 effect on IKK$^*$</td>
<td>$\beta$</td>
<td>0.02 min$^{-1}$</td>
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</table>

Table 3.2.1: Parameters used in the model.

The dynamical variable of most importance in the model is $N_n$, the nuclear NF-κB concentration. The first term in equation 3.2.1 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 protein, as IκB sequesters the transcription factor in the cytoplasm. The negative term models the decrease of the nuclear concentration due to sequestration by the IκB and subsequent export into the cytoplasm. At the timescales we are interested in there is no significant production or degradation of NF-κB [Krishna et al., 2006]. The mRNA level of IκB is regulated through a sigmoidal function of NF-κB, given by $N_n^2 / N_n^2 + K^2$. Here we assume a weak cooperativity in NF-κB activating transcription of IκB with a Hill coefficient two.

The rate of IκB protein increase is linearly proportional to the level of mRNA, $I_m$. The rate of decrease in IκB is controlled by both an IKK-independent degradation, $\alpha$, and an IKK-dependent degradation, $\alpha$. The rate of IκB degradation is proportional to both IKK activity and the concentration of complexes formed between IκB and cytoplasmic NF-κB, $N_c \propto IKK[I\kappa B0 : N_c]$. This saturated degradation has earlier been discussed in section 2.1 on page 13. Assuming that reaction rates for complex formation are much faster than the nuclear import/export and IκB degradation, the concentration of $[I\kappa B0 : N_c]$ can be derived to be $(1 - N_c)/(1 + I\kappa B0)$. The model parameters has been chosen to reproduce the desired experimental features.
CHAPTER 3. OSCILLATIONS IN NUCLEAR NF-κB ACTIVITY

A20-like Proteins Change the Period of Nuclear NF-κB Oscillations

![Diagram of network and model responses](Figure 3.2.2: A20-like proteins change the period of nuclear NF-κB oscillations. A) Schematics of the nested network where the feedback from A20 onto IKK is marked by the yellow arrow. B) Model response of NF-κB nuclear/cytoplasmic ratio and IKK activity for A20 knockout (blue), Wild-type (red) and 56-fold A20 feedback (green), compared to wild-type (green). C) Heatmap recording the period of the nuclear NF-κB oscillations, the steady state level and the amplitude of the oscillation, as a function of the changing parameter σ. A20-like proteins change the period of nuclear NF-κB oscillations and lower the stead state level.

The basic response of the model to a continuous stimulation of TNF is oscillations of nuclear NF-κB with a time period of about 100 minutes. The original wild type response as well as IKK and A20 knockout matches the experimental observations, see figure 3.2.2. Thus, the basic response (criterion 1 and 2) is correctly reproduced by the model.

In agreement with the literature [Lee et al., 2000, Werner et al., 2008, Trompouki et al., 2003, Brummelkamp et al., 2003, Kovalenko et al., 2003, Enesa et al., 2008] we see a substantial lowering of the end level of nuclear NF-κB in wild-type cells compared to A20-knockout cells, figure 3.2.2 (B). Additionally we find that A20-like proteins are able to adjust the period of nuclear NF-κB oscillations in the range from 0.5 to 2 hours, figure 3.2.2 (C). We observe that as the A20 feedback gets stronger the period of the oscillations is increased and the amplitude decreased.

Other Parameters Affecting the Period of the Nuclear NF-κB Oscillations

In figure 3.2.2 we used σ (in eq. 3.2.2) as a proxy for the A20 feedback strength. We will in the following investigate other parameters in the model to see what effect they would have on the period of the oscillations. The following parameters are varied: A20 feedback onto IKK (σ, fig. 3.2.2), IKK self inactivation (μ), level of TNF induction, A20 transcription rate (t_A) and A20 constitutive production (p). They have all been varied in a range around the wild-type parameter value (labeled ‘1’ in fig. 3.2).

It is seen how both A20 feedback strength (σ) and A20 transcription rate (t_A) are able to increase the period, and likewise IKK self-inactivation, figure 3.2. Only the level of TNF has no effect on the period of the nuclear oscillations as the IKK cycle tightly controls the level of protein degradation.

Comparing the change in oscillation period with the change in amplitude and steady state level, figure 3.2.4 (A-B), we observed that A20 constitutive level (p) is able to bring down the level of IKK activity and the amplitude of the oscillations, but is only able to
3.2. RESULTS

Figure 3.2.3: Change in the period of nuclear NF-κB oscillations due to variation in parameters in the model: $\sigma$ is the $A20$ feedback strength, $\mu$ determines IKK self inactivation, TNF is the level of TNF the system is stimulated with, $t_A$ is the transcription rate for $A20$ mRNA and $p$ is constitutive production of $A20$ mRNA.

change the period of the oscillations in the presence of NF-κB induced $A20$, when increased sufficiently. Constitutive $A20$ expression in $A20$ knockout cells produced damped oscillations with a high steady state but not dependent on the rate of constitutive production.

Figure 3.2.4: Change in the amplitude, steady state and spikiness of nuclear NF-κB oscillations due to various different parameters in the model: $\sigma$ is $A20$ feedback strength, $\mu$ determines IKK self inactivation, TNF is the level of TNF the system is stimulated with, $t_A$ is the transcription rate for $A20$ mRNA and $p$ is constitutive production of $A20$ mRNA. Black triangles in (C) is the transcription rate for $IκBα$.

Lastly, we take a brief look at the spikiness of the oscillations, reported in [Krishna et al., 2006]. The spikiness of the oscillations can be quantified by

$$Z = \frac{(\max(N_n) - \min(N_n))}{\text{mean}(N_n)}.$$  

Oscillations with $Z > 2$ are termed spiky and oscillations with $Z < 2$ are termed soft oscillations, as in [Krishna et al., 2006]. None of the above mentioned parameters are able to drive the oscillations above $Z = 2$, except for the transcription rate of $IκB$ (black triangles in figure 3.2.4 (C)). This is mainly due to the low amplitude and relatively broad oscillations generated by the model. Note how the transcription rate of the two inhibitors $IκBα$ (black triangles in (C)) and $A20$ (blue triangles) has the opposite effect on the oscillation characteristics. Where a higher transcription rate of $A20$ will
bring down the amplitude and thus the spikiness of the oscillations does an increased transcription rate of IκBα enhance the spikiness and the amplitude (result not shown) of the nuclear oscillations. This highlights the difference in the feedback from the two proteins, where A20 is reducing the effect of IKK and thus the release of NF-κB is the inhibitory effect from IκBα driving the oscillations.

Discussion

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 a cell proliferating and protect it 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 [Hoffmann et al., 2002] which on a population level appears as damped oscillations. Such temporal control can allow for selective gene activation [Werner et al., 2005, Ashall et al., 2009]. Given that the NF-κB temporal response is of high importance and in single cells is primarily regulated by ‘nested’ negative feedback loops we here investigated the role of the A20-like negative feedback.

A novel finding of our investigation suggests that in single cells not only does A20 bring down the resting level of the nuclear oscillations, it can also adjust the period of the oscillations by changing the IKK profile. Thus, A20-like proteins together with IκB allow for independent tuning of frequency through A20-like negative feedbacks (in contrast, IκBα controls the spikiness of the oscillations). This combination of nested feedback loops covers a wide variety of temporal responses where one can access both sustained oscillations and damped oscillations with low or high frequency.

Figure 3.2.5: A, Model simulations with the full NF-κB model presented in [Werner et al., 2008]. Each simulation is performed with a different strength of the A20 feedback onto IKK. B, Period is measured as the time between first and second peak and displayed against the strength of A20 feedback onto IKK. An increase in the timing of the second peak is seen as the feedback of A20 onto IKK becomes stronger.

If the biphasic response as seen in bulk experiments is a result of the population average of single cells with oscillating NF-κB, then, in bulk experiments, A20 will exhibit its effect by affecting the timing of the second phase onset. By using the model presented in [Werner et al., 2008] we can show how the period, measured as the time
between first and second peak, is modulated by the A2o feedback onto IKK, figure 3.2.5. Thus a specific prediction would be that, as the timing between first two peaks is shorter, the second phase should start earlier in A2o knockout cells (figure 3.2.2 (B)).

Our findings lead to a clear prediction that in single cells decreasing the coupling between A2o-like proteins and IKK should lead to higher frequency oscillations in NF-κB. This can be experimentally tested by knocking down A2o-like proteins with siRNA. 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.

3.3 Potential Physiological Role of Oscillations

However, we still do not know whether oscillations in nuclear transcription factor activity have a functional role in adjusting downstream gene expression. It is interesting that saturated degradation typically produces quite spiky oscillations, as seen in [Krishna et al., 2006], which have the advantage that the signaling molecule can achieve high levels without having to be produced at a high rate all the time. I will in this last part of the chapter elaborate on the question of what these oscillations are useful for.

In some systems periodicity is an obvious requirement. A periodic spatial pattern is clearly necessary for proper somite spacing and temporal oscillations in Wnt and Notch targets are a way of generating the spatial periodicity [Cooke and Zeeman, 1976, Pourquie, 2003]. Circadian clocks in cyanobacteria are useful for entraining metabolism, photosynthesis, cell division and global gene expression to the day-night cycle [Yang et al., 2010]. However, in NF-κB and p53 it is not obvious that the oscillations per se are important for the physiological response. For example, it has been suggested that p53 pulses might be a byproduct of pulsatility in ATM, an upstream regulator of p53 required for proper DNA damage repair [Batchelor et al., 2009].

From the opposite angle, what benefit could oscillations provide in helping NF-κB and p53 produce gene expression patterns specific to distinct stimuli? One idea is that signals with complex temporal variation contain more information than steady-state signals and therefore can control downstream genes more subtly [Krishna et al., 2006, Kobayashi and Kageyama, 2009, Batchelor et al., 2009].

Encoding Information in Oscillatory Signals

Steady-state signals have a single adjustable characteristic, the level of the signal, while oscillations have many -- average, amplitude, time period, spikiness, spike width, spike symmetry, figure 3.3.1. Oscillations in NF-κB or p53 could thus encode more information than steady-states about which stimulus was triggering the system provided: (i) different stimuli affect different parameters, and (ii) changing different parameters affects oscillation characteristics differently.

(i) In the p53 system, we know that different stimuli affect different sets of parameters. DNA damage affects Mdm2 activity and stability, hypoxia additionally alters the transcription rate of Mdm2, while other triggers like Nutlin only change the binding strength between p53 and Mdm2 (see references in [Hunziker et al., 2010]). In the NF-κB model, many triggers act through the IKK level which affects the degradation rate of IκBα, but different stimuli produce different profiles of IKK and thereby NF-κB [Werner et al., 2005, Lee et al., 2009]. (ii) Figure 3.3.1 (B-E) show that for the simple model of NF-κB described in section 2.1, changing different parameters does indeed
affect oscillation characteristics differently. The plots show that there are parameter regimes where one of the three characteristics, time period, average and peak, can vary while the other two remain constant. Peak levels can be varied independently of time period and average by changing $N_{tot}$ when oscillations are smooth. Similarly, average levels can be varied independently of the other two by changing $k_c$ when oscillations are smooth. Finally, time period can be varied while average and peak remain constant by changing $\delta$ when oscillations are spiky. For all the parameter ranges shown, we also found that increased spikiness is correlated with larger time periods, lower averages and asymmetry of the spike shape. However, not all characteristics can be independently varied because there are correlations. For example, spikiness is correlated with larger time periods, lower averages and asymmetry of the spike shape. Experiments have also shown that characteristics of the temporal profile of nuclear NF-κB concentration, e.g. the steepness of the initial increase and the later decline, are under control of different regulators [Werner et al., 2008]. The sharp transition from spiky to smooth oscillations in figure 3.3.1 (B) occurs when the degradation rate ($\alpha_{IKK}$) exceeds the production
Decoding Information from Oscillatory Signals

Next, it is necessary that different genes should respond to different characteristics of the oscillations. We consider the simplest case where an oscillating TF binds to a single operator site, figure 3.3.2 (A). Two parameters describe the binding: the association ($k_{on}$) and dissociation ($k_{off}$) rate constants. Figure 3.3.2 (A) shows that the expression of a gene, $G_{fast}$, with sufficiently large $k_{on}$ and $k_{off}$, will closely follow the oscillations. We make the (conservative, for NF-$\kappa$B and p53) assumption that the maximal concentration of the TF is 100-fold the operator concentration. Gene $G_{fast}$ has $k_{on} = 0.1 \text{ min}^{-1}$ per operator site, $k_{off} = 0.3 \text{ min}^{-1}$ and follows the oscillations of the TF closely (green). $G_{slow}$ has $k_{on} = 0.1 \text{ min}^{-1}$ per operator site, $k_{off} = 0.003 \text{ min}^{-1}$ and its peak expression follows the average level of the oscillations. Note the vastly different responses in the two cases. (B) $G_1$ ($k_{on} = 0.1 \text{ min}^{-1}$ per operator site, $k_{off} = 0.03 \text{ min}^{-1}$) and $G_2$ ($k_{on} = 0.3 \text{ min}^{-1}$ per operator site, $k_{off} = 0.06 \text{ min}^{-1}$) respectively activate and inhibit a third output gene $G$. The average expression of this output gene has a maximum for a certain "resonance" frequency rate of the inhibitor protein ($k_c$). Similarly, the transitions in figure 3.3.1 (C-E) are due to the balance between these two rates. Similar behavior is seen in the p53 model (and the Wnt model not mentioned in details here), so one can conclude that an oscillatory signal produced from a simple negative feedback loop with saturated degradation can indeed encode more information than steady-state signals.

3.3. POTENTIAL PHYSIOLOGICAL ROLE OF OSCILLATIONS

Figure 3.3.2: Decoding information from oscillatory signals. (A) Regulation of gene expression by a transcription factor (TF) that associates to a single operator site with a rate constant $k_{on}$ and dissociates with a rate constant $k_{off}$. Gene $G_{fast}$ has $k_{on} = 0.1 \text{ min}^{-1}$ per operator site, $k_{off} = 0.3 \text{ min}^{-1}$ and follows the oscillations of the TF closely (green). $G_{slow}$ has $k_{on} = 0.1 \text{ min}^{-1}$ per operator site, $k_{off} = 0.003 \text{ min}^{-1}$ and its peak expression follows the average level of the oscillations. Note the vastly different responses in the two cases. (B) $G_1$ ($k_{on} = 0.1 \text{ min}^{-1}$ per operator site, $k_{off} = 0.03 \text{ min}^{-1}$) and $G_2$ ($k_{on} = 0.3 \text{ min}^{-1}$ per operator site, $k_{off} = 0.06 \text{ min}^{-1}$) respectively activate and inhibit a third output gene $G$. The average expression of this output gene has a maximum for a certain "resonance" frequency

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Chapter 3. Oscillations in Nuclear NF-κB Activity

\( G_{\text{slow}} \) is therefore an average-detector.

The slightly more complex circuit of figure 3.3.2 (B) is a frequency-detector. Two genes with 2-fold different \( k_{\text{off}} \) values respectively activate and inhibit a third, output gene. \( G_1 \) has a \( k_{\text{on}} = 0.1 \text{ min}^{-1} \) per operator site and a \( k_{\text{off}} = 0.03 \text{ min}^{-1} \) and is activating \( G_3 \). \( G_2 \) has a \( k_{\text{on}} = 0.1 \text{ min}^{-1} \) per operator site and a \( k_{\text{off}} = 0.06 \text{ min}^{-1} \) and inhibits \( G_3 \):

\[
dG/dt = k \frac{G_1}{1 + G_1/K_1} \left( \frac{1}{1 + (G_2/K_2)^2} \right) - \gamma G
\]

For this circuit, we can find parameter values for the \( dG/dt \) equation such that the average expression of \( G \) has a maximum (‘resonance’) when the time period is around \( T=150 \text{ min.} \), when the input is spiky square-pulse oscillations (green curve). The position of this maximum can be tuned by varying \( k_{\text{off}} \) values of \( G_1 \) and \( G_2 \). Away from this resonance, especially for larger frequencies, the output falls dramatically. Interestingly, the spiky nature of the input oscillations is very important for this. With the same parameter values, when the input is smooth sine-wave oscillations of the same amplitude the response is much weaker (blue curve). This circuit is therefore a spikiness-detector as well.

### 3.4 Discussion

Some of the interesting questions this discussion raises for future research on oscillatory control are related to encoding information in oscillations, such as: which parameters, and which characteristics of oscillations, do different stimuli affect in the NF-κB and p53 systems? Do some triggers of NF-κB affect processes other than the degradation of \( I_B \)? How does varying these parameters affect oscillations in more detailed models? Can additional feedback loops enhance the encoding abilities of oscillations?

Also relating to decoding information from oscillations: Can other decoding circuits be constructed to count, say, the number of spikes in a signal, or distinguish between symmetry and asymmetry, or other characteristics of oscillations? What mechanisms can make the frequency response of a gene circuit sharper and more tunable? Do any such circuits exist downstream of NF-κB or p53?

The ideal experiment to evaluate the necessity of oscillations would require the ability to control the frequency, number and width of spikes produced when NF-κB or p53 is triggered, and to see how this affects the physiological response. Exactly such experiments have shown that varying the frequency of oscillations in calcium signaling [Zhu et al., 2008] and hormone secretion [Ciccone et al., 2010] changes the physiological response. Another example of frequency dependence is in hormone regulation. For instance, high frequency pulses in GnRH result in secretion of LH, while low frequency pulses cause secretion of FSH, and thereby a different frequency response. Similar experiments on the NF-κB system are just beginning to be actualized, and have reported some dependence of gene expression on frequency of oscillations [Ashall et al., 2009]. Other experiments have shown the opposite, that expression of some NF-κB targets is unaffected in non-oscillating mutants [Barken et al., 2005]. These results are not necessarily contradictory, but until more comprehensive experiments become feasible for the NF-κB and p53 systems it might be useful to examine more carefully the genetic circuits downstream of these TFs. A frequency-detector circuit downstream of NF-κB or p53 would be a strong clue that oscillations are important for the physiological response.
We have now looked at models of the nuclear NF-κB response and potential functional roles of oscillations in transcription factor activity. In the following chapter I will go yet another step in detail with the NF-κB signaling system and look at the actual transcription factor - DNA interaction. Experimental work suggests that ItκBo is capable of actively removing NF-κB from the DNA site. This effect has earlier been studied by [Zabel and Baeuerle, 1990] and we here call it *stripping*. In the following chapter we preform a computation analysis of the stability of the transcription factor (TF)•DNA complex and the effect of stripping.


### 4.1 Introduction

The half-life of the NF-κB•DNA complex has during the last decades been measured by several groups using different techniques, all giving contrary answers to the question of the stability of the TF•DNA complex. In 1990 [Zabel and Baeuerle, 1990] estimated the half-life of the TF•DNA complex to be 45 min measured *in vitro* by Electrophoretic Mobility Shift Assay (EMSA)\(^1\). Using the same technique [Phelps et al., 2000] published a half-life (\(T_{1/2}\)) of 20 sec of the p50/p65 heterodimer. The first *in vivo* studies were done in 2006 by [Bosisio et al., 2006] using Fluorescence Recovery After Photobleaching (FRAP) analysis to estimate a complex half-life of 1 sec. This wide span from 1 sec to 45 min in the measured half-life of the TF•DNA complex may be due to the different techniques applied, a difference in the kinds of DNA used and even the NF-κB dimer measured. We have in paper 1 used Biacore to measure the *in vitro* half-life for NF-κB(p50/p65) binding to IgκB sites to be 40 sec at 37°C.

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\(^1\)Biochemical technique used to study protein-DNA interactions. Also called gel shift assay
The dynamics of the transcription factor NF-κB has as described in the previous chapters been modeled a number of times capturing the nuclear localization of NF-κB in response to a variety of stimuli [Hoffmann et al., 2002, Lipniacki et al., 2004, Krishna et al., 2006] and (paper 3). However, these models do not describe NF-κB - DNA interaction. The models are based on mass action kinetics, driven by reaction rates achieved from biochemical experiments. The tuning of the models is done by fitting the output of the model to relevant experimental data. Earlier proposed models of TF - DNA interaction are based on equilibrium binding probability considerations [Gerland et al., 2002] but do not capture the kinetics of the system.

We here present a mass action kinetic based model of the NF-κB - DNA interaction, analyzing the role of IκBα mediated dissociation of the TF-DNA complex in the presence of both target and off-target DNA. Target DNA is the DNA which has specific binding sites for the transcription factor leading to gene transcription, whereas off-target DNA is non-specific and does not result in gene transcription.

We imagine that active stripping of IκBα from the DNA sites is important for fast dynamics when the TF-DNA complex half-lives are long. If the TF-DNA complex half-life is 1 sec or less, as determined by [Bosisio et al., 2006] it does seem surprising if stripping has an important effect for the dynamics of the complex. However, the short half-life measured may apply to off-target DNA and not the DNA interaction which lead to gene transcription.

![Figure 4.1.1: A: Real time NF-κB:DNA binding measured by Biacore. At t = 0 different concentrations of IκBα injected into the system disrupting the NF-κB•DNA complex. A schematic of the binding events is shown below the graph. B: Model simulations of IκBα mediated dissociation of the NF-κB•DNA complex. Simulations are performed by letting NF-κB associate to the 100nM target DNA and at time = 0 allow a constant flow of IκB through the system. The concentration of IκBα is given by the color code of (A). C: Plot of the dissociation rate constant determined from 4 independent experiments, as described in A, as a function of IκBα concentration. The slope of the line (10^6 M^-1 s^-1), which is the pseudosecond-order rate constant for active dissociation, indicates that IκBα-enhanced dissociation is highly efficient. Data from (paper 1).]
Surface Plasmon Resonance (SPR) Experiments

Real-time binding and dissociation of the TF to DNA was monitored by SPR using Biacore 3000. Biotinylated α-site DNA was bound to a streptavidin chip (t = 0). NF-κB(p50 (19–363)/p65(1–325)) was allowed to associate with the DNA until an equilibrium was reached, approximately at t = 100 s, see figure 4.1.1 (B). Varying concentrations of IxB were then injected through a second sample loop (co-inject experiment), and the dissociation rate constant (k_d) of NF-κB from the DNA was measured. All experimental work has been performed by S. Bergqvist and V. Alverdi.

4.2 Results

Model of NF-κB:DNA Interaction

Our first aim is to reproduce the dissociation data achieved by Biacore (figure 4.1.1 (A)) and secondly to analyze the effect of multiple DNA types on the stability of the target complex. Here I will go straight to the model of both target and off-target DNA, where the model of only target DNA is represented by just setting the off-target interaction rates to zero. The output of such model simulations are shown in figure 4.1.1 (B), illustrating the active dissociation of the NF-κB:DNA complex at different concentration of IxBα.

We have built a simple ordinary differential equation (ODE)-based model to address the question of the role of IxBα mediated stripping of the TF from the DNA in the presence of both target and off-target DNA. The model contains the components NF-κB, target DNA, off-target DNA, IxBα and their complexes. NF-κB can bind to the DNA (k_2), unbind the DNA (k_7) and form a complex with IxBα (k_1). Additionally IxBα can bind to the NF-κB•DNA complex (k_3) and dissociate the complex forming a free NF-κB-IxBα complex (k_4). Likewise NF-κB can interact with the off-target DNA, see figure 4.2.1. The equations for the model are listed below:

Figure 4.2.1: Schematic drawing of NF-κB - DNA interaction with both target DNA and off-target DNA

*Streptavidin is a protein purified from bacteria which has an extremely high affinity for biotin, independently of pH, temperature, detergents etc.*
\[
\frac{dN}{dt} = -k_1 N \cdot I + k_{-2} D_t N - k_2 N \cdot D_t + k_{-5} N \cdot D_{off-t} - k_5 N \cdot D_{off-t}
\]
\[
\frac{dNI}{dt} = k_1 N \cdot I + k_4 N D_t - k_7 N D_{off-t}
\]
\[
\frac{dD_t}{dt} = k_{-2} D N - k_2 N \cdot D_t + k_{-4} N D_t
\]
\[
\frac{dDN_t}{dt} = k_2 N \cdot D_t - (k_{-2} + k_3 \cdot I) N D_t + k_{-3} N D_t
\]
\[
\frac{dDNI}{dt} = k_3 N D_t \cdot I - (k_{-3} + k_{-4}) N D_t
\]
\[
\frac{dD_{off-t}}{dt} = k_{-5} N D_{off-t} - k_5 D_{off-t} \cdot N + k_{-7} N D_{off-t}
\]
\[
\frac{dDN_{off-t}}{dt} = k_5 D_{off-t} \cdot N - (k_{-5} + k_6 \cdot I) N D_{off-t} + k_{-6} N D_{off-t}
\]
\[
\frac{dDNI_{off-t}}{dt} = k_6 N D_{off-t} \cdot I - (k_{-6} + k_{-7}) N D_{off-t}
\]

where NI is the NF-κB:IkB complex. DN is the NF-κB:DNA complex and DNI is the NF-κB:IkB:DNA complex both for target DNA (t) and off-target DNA (off-t).

**Model Parameters**

The on- and off-rates governing the NF-κB-DNA complex formation have been determined by SPR measurements (Biacore) and can be found in table 4.2.1. We are using the rates for the p50/p65 heterodimer as this is the more common and well studied dimer. The IkBo-NF-κB complex formation is described by previous models from which the association rate was found [Werner et al., 2005]. IkBo-NF-κB dissociation is set to be zero as it is considered to be very slow and not relevant in the time scale we are looking at. IkBo-NF-κB dissociation is in the cell additionally mediated by the inhibitor kinase (IKK) but we are here only modeling the DNA interaction leaving the stimulus induced dynamics to later in this chapter. IkBo association to the NF-κB:DNA complex and subsequent dissociation rates have been calculated from the measured stripping rate by assuming that complexes are at equilibrium. All rates are assumed to be the same for the target and off-target DNA, except for NF-κB dissociation rate.

We assume that the amount of DNA in the nucleus is large compared to the number of NF-κB molecules and most probably a large number of DNA sites are accessible for NF-κB to interact with. Some of the sites will lead to recruitment of the promoter and gene transcription (target DNA) but other sites will only lead to brief interaction with NF-κB and no transcription initiation (off-target DNA), figure 4.2.1 (box). The accessibility of the DNA site depends on chromatin structure and other factors which I will not touch upon here.

In order to use physiological relevant concentration of DNA sites we have estimated the number of target DNA sites to be 1000. This estimation is based on roughly 200 genes being transcribed by NF-κB, all having 5 DNA sites. The 1000 DNA sites have been re-calculated to a concentration of 20nM. The amount of off-target DNA in a cell is currently not defined in the literature and we are thus varying this quantity in the different simulations. The TF can most likely interact with off-target DNA sites in the μM range at least, and thereby 100-1000 times the target DNA. This estimate is in
4.2. RESULTS

<table>
<thead>
<tr>
<th>Rate</th>
<th>Reaction</th>
<th>DNA type</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_1$</td>
<td>NF-κB–IκB association</td>
<td>target DNA</td>
<td>0.5 $\mu M^{-1} sec^{-1}$</td>
</tr>
<tr>
<td>$k_2$</td>
<td>NF-κB–DNA association</td>
<td>target DNA</td>
<td>1.2 $\mu M^{-1} sec^{-1}$</td>
</tr>
<tr>
<td>$k_{-2}$</td>
<td>NF-κB–DNA dissociation</td>
<td>target DNA</td>
<td>0.007 $sec^{-1}$</td>
</tr>
<tr>
<td>$k_5$</td>
<td>NF-κB–DNA association</td>
<td>off-target DNA</td>
<td>1.2 $\mu M^{-1} sec^{-1}$</td>
</tr>
<tr>
<td>$k_{-5}$</td>
<td>NF-κB–DNA dissociation</td>
<td>off-target DNA</td>
<td>0.7 $sec^{-1}$</td>
</tr>
<tr>
<td>$k_3$</td>
<td>IκB–NF-κB : DNA association</td>
<td>target DNA</td>
<td>1.0072 $\mu M^{-1} sec^{-1}$</td>
</tr>
<tr>
<td>$k_{-3}$</td>
<td>IκB–NF-κB : DNA dissociation</td>
<td>target DNA</td>
<td>0.005 $sec^{-1}$</td>
</tr>
<tr>
<td>$k_4$</td>
<td>IκB:NF-κB–DNA dissociation</td>
<td>target DNA</td>
<td>0.7 $sec^{-1}$</td>
</tr>
<tr>
<td>$k_6$</td>
<td>IκB–NF-κB : DNA association</td>
<td>off-target DNA</td>
<td>1.0072 $\mu M^{-1} sec^{-1}$</td>
</tr>
<tr>
<td>$k_{-6}$</td>
<td>IκB–NF-κB : DNA dissociation</td>
<td>off-target DNA</td>
<td>0.005 $sec^{-1}$</td>
</tr>
<tr>
<td>$k_7$</td>
<td>IκB:NF-κB–DNA dissociation</td>
<td>off-target DNA</td>
<td>0.7 $sec^{-1}$</td>
</tr>
</tbody>
</table>

Table 4.2.1: Reaction rates used in the DNA interaction model

agreement with previous estimates, [Mirny and Wunderlich, 2009]). The affinity of the target sites are given by Biacore experiments, with a $K_D$ of 5.8 nM and a half-life of $\sim 100 \text{ sec}$ (paper 1) at 25°C. The off-target sites are in the simulations given a $K_D$ of 580 nM and a half-life of $\sim 1 \text{ sec}$ to match the half-life found by [Boisso et al., 2006]. The on-rate for NF-κB binding to the DNA is assumed to be the same for both DNA types and IκBα is assumed to equally bind and strip NF-κB from the target and the off-target DNA.

Stripping is introduced in the model by multiplying a factor to the association reaction of IκBα with the NF-κB•DNA complex. IκBαNF-κB complex dissociation is mediated directly after IκBα is bound to the NF-κB•DNA complex. Stripping has units of $\mu M^{-1} sec^{-1}$ and illustrates the effective NF-κB•DNA dissociation rate. At an IκBα flux of 10$nM/sec$ the stripping factor is $1\mu M^{-1} sec^{-1}$ which is equivalent to what we determined experimentally by real-time binding and dissociation experiment measured using SPR, figure 4.1.1 (A and C) in paper 1.

Prolonged Complex Half-lives in the Presence of Off-target DNA

First we analyze the effect of off-target DNA on the dynamics of the target DNA-NF-κB complex, figure 4.2.2, keeping the amount of target DNA at 20nM. Figure 4.2.2 (A) shows the concentration of the target complex in the presence of four different concentrations of off-target DNA. The association of the TF•DNA complex happens very fast and we let the system equilibrate before adding IκBα to the simulation. At time = 0 IκBα is added to the system and the level of target complex is immediately reduced. The half-life of the complex is the time at which half of the maximum concentration of the complex is left in the system. Computationally this has been measured by recording the concentration of formed complex right before IκBα is added to the system and measuring the half-life as the time at which the amount of complex is equal to half of this maximum concentration. The TF – DNA binding is always taken to equilibrium before IκBα is added to the system.

The amount of target complex formed is strongly reduced the more off-target DNA present in the system. The off-target DNA is competing for the free NF-κB thus forming less target complex. This additionally prolongs the half-life of the target DNA complex as NF-κB bound to the off-target DNA is able to rebind to the target DNA once released from the off-target sites. I will discuss this in further detail on page 42. Figure 4.2.2 (B)
CHAPTER 4. STRIPPING - A MECHANISM OF GENE REGULATION

Figure 4.2.2: A: Complex formation between NF-κB and target DNA in the presence of different concentrations of off-target DNA. B: The half-life of the target complex is greatly prolonged in the presence of large amounts of off-target DNA along with a reduced total amount of target complex formation.

displays the half-life of the individual simulations as a bar graph showing how the half-life is greatly prolonged the more off-target DNA present in the simulation.

Stripping Breaks Down the NF-κB:DNA Complex at High and Low Concentrations of Off-target DNA

Figure 4.2.3: Stripping transcription factors off the DNA. A: Complex formation between NF-κB and target DNA in the presence of different concentrations of off-target DNA. Stripping reduce the half-life of the target DNA complex. B: The half-life of the target complex is dramatically reduced in the presence of large amounts of off-target DNA when IκBα is binding to the NF-κB•DNA complex and actively removing NF-κB.

Changing the rates so that IκBα can associate with the NF-κB•DNA complex and subsequently dissociate NF-κB from the DNA site reveals a large change in system dynamics. The long complex half-lives observed above (figure 4.2.2) are all dramatically reduced and even shorter half-lives are found in the presence of larger amounts of off-target DNA, figure 4.2.3 (A). NF-κB is now captured by IκBα already on the DNA site which primarily prevents its self-release and thus the ability to re-bind to tar-
get DNA. Stripping is specifically important at high concentrations of off-target DNA where the half-life of the target complex otherwise is very prolonged, figure 4.2.4 and figure 4.2.4 (A, top, left corner).

Simulations are additionally performed at varying effective stripping strengths, from zero to $8\mu M^{-1} sec^{-1}$, figure 4.2.4 (A). A stripping factor of zero is equivalent to not letting IκBα interact with the NF-κB•DNA complex and thereby NF-κB has to dissociate from the DNA on its own. Very short half-lives are found with a stripping factor of 1 or more. Interestingly, short half-lives are also seen in the presence of high amounts of off-target DNA as a high amount of off-target DNA traps the accessible NF-κB leaving minimal NF-κB for target complex formation. The small amount of target complex is at high efficiency being dissociated by the constant IκBα protein flow. NF-κB bound to the off-target DNA is also removed by IκBα and is therefore not able to rebind the target DNA. This leads to the short complex half-life seen in the top right most part of the heat map in figure 4.2.4 (A) and figure 4.2.2. Long complex half-lives are seen at high concentrations of off-target DNA and no stripping. The amount of off-target DNA does, on the other hand, not affect the half-life of the target DNA complex when IκBα is stripping NF-κB off the DNA, right most part of (A) in figure 4.2.4.

Figure 4.2.4: A: Measuring the half-life of the target DNA:NF-κB complex at different concentrations of off-target DNA and at different effective stripping factors. B-C: Half-life of the productive DNA at different concentrations of off-target DNA and for different affinities ($K_D$) of the target DNA. [NF-κB] = 100 nM, [IκBα] flux = 10 nM sec$^{-1}$, [target - DNA] = 20nM. B: IκBα can only bind to NF-κB when free in the nucleus, C: IκBα mediated dissociation of the NF-κB•DNA complex. White circles indicate the parameter values used for the line plot simulations in figure 4.2.2 and 4.2.3.

Stripping Terminates the NF-κB:DNA Complex at High and Low Concentrations of Off-target DNA Independent on the TF Affinity for the Target DNA

Next we analyze the effect of different DNA affinities on the half-life of the target DNA complex.

The affinity for the DNA site is a measure of how easily the TF is released from the DNA after binding. The dissociation constant is given as $K_D = \frac{\text{dissociation rate}}{\text{association rate}}$. High affinity sites have a small off-rate of the substrate and thus a small $K_D$, leaving the TF longer at the DNA site. In figure 4.2.4 (B, C) the amount of off-target DNA vs the dissociation constant ($k_D$) of the target TF•DNA complex is changed, having a reference $K_D$ from the Biacore experiments (paper 1) of 5.8nM. These simulations
are done without and with stripping, (B) and (C), respectively. Interestingly, the complex half-life is found to be independent of the affinity of NF-κB for the target DNA, if NF-κB is being actively removed from the DNA through IκBα mediated stripping, figure 4.2.4 (C). This is seen as a short complex half-life (red) for all concentrations of off-target DNA and all dissociation constants of the target DNA. A short target TF•DNA complex half-life is only observed at low amounts of off-target DNA and with very high dissociation rates (low affinity) of the target DNA, if IκBα is not able to strip NF-κB off the DNA, see lower right corner in 4.2.4 (B).

Rates and Stability Considerations
The stability of the target-DNA complex is very dependent on the rebinding of NF-κB released from both target and off-target DNA. Disallowing the rebinding of NF-κB from either of the two DNA types will dissociate both complexes even before IκBα enters the system. The half-life of the target complex is indeed prolonged by the presence of off-target DNA as this DNA traps the free NF-κB from the IκB binding in solution, allowing it to rebind other DNA’s. The IκB flow through the system is 10nM/sec and the IκB bind the DNA bound NF-κB with a slightly smaller rate than binding to the free NF-κB, 1.0072μM⁻¹sec⁻¹ vs 1.2μM⁻¹sec⁻¹. Binding between IκBα and NF-κB is 0.5μM⁻¹sec⁻¹ which is slower than the re-binding to the DNA. Once the TF is off the DNA is it more likely to bind new DNA than the inhibitor protein IκBα, dependent of the present concentrations of DNA and IκBα protein. If IκBα is allowed to bind directly to the DNA complex is the effective binding rate much larger and the TF does not get the choice of rebinding other DNA molecules.

The half-life of the target DNA complex is 30 min. at 2μM off-target DNA and an off rate from the off-target DNA of \( k_{-5} = 0.07 \), 20nM target DNA and no stripping. The half-life of the off-target DNA is under these conditions 10 min. If the amount of off-target DNA is lowered to 0.2μM is the half-life of the target DNA now 15 min. and of the off-target DNA 3.4 min., keeping all other rates constant.

Including DNA Interaction in the NF-κB Model
We have now analyzed the role of active IκBα mediated dissociation of the TF•DNA complex in a small model where there is no cellular relocation and the endpoint for the transcription factor activity is the binding to IκBα. We will next introduce the DNA interaction module in a larger dynamic model of the full NF-κB response, earlier presented by [Werner et al., 2008], where the flux of IκBα is part of the model dynamics and the IκBα-NF-κB complex is dissociated by TNF induced IKK activity.

Including the DNA interaction module in the full [Werner et al., 2008] NF-κB model (figure 4.2.5 (A)), is not completely trivial. The [Werner et al., 2008] model has been developed without DNA interaction and the present rates and interactions are in some way compensating for the lack of DNA. Rates in the model have been set by both experimental measurements, calculations and model fit, to allow for a detailed model reproduction of the experimentally generated data. When introducing the DNA interaction module just as it is, and not making any changes in reaction rates, the response is slowed down dramatically and NF-κB is now completely trapped in the nucleus. Again, this is because the model dynamics has been fittet to the data without the DNA interaction part. To properly include DNA interaction in the existing NF-κB model we must carefully consider the parameters and which we can change and fine-tune accordingly,
in the aim of reproducing the observed nuclear NF-κB activity reported [Hoffmann et al., 2002].

Recently [Giorgetti et al., 2010] has predicted that NF-κB does not bind cooperatively to the DNA as earlier believed, but with a Hill coefficient of 1.1. The Hill coefficient has been 3 in the earlier model ([Werner et al., 2008] and previous) and we have in (paper 3) used h=2. We have, based on this latest finding chosen to bring the Hill coefficient in the model. We have additionally brought down the NF-κB induced transcription rate due to the small amounts of target DNA. A high transcription rate increases the export of NF-κB from the nucleus as more IκBα proteins are synthesized. All other reaction rates are the same as published in [Werner et al., 2008].

The DNA interaction module has the same reaction rates as the small model presented above, only is the NF-κB off-rate from the off-target DNA 10 times larger than the target DNA and not 100 times as in the small model.

Applying these few changes reproduces the data nicely, figure 4.2.5 (C), displaying the DNA bound NF-κB concentration of both target and off-target DNA, as this is the measure recorded by EMSA in [Hoffmann et al., 2002]. All analysis performed on the small interaction module above were only measuring the activity of the target-DNA complex.

We have thus decided only to change the transcription rate of the inhibitor protein and the Hill coefficient in the original model. Lowering the dissociation rate from the off-target DNA is equivalent to a half life of 10 sec in the small model. From the small model scan of different dissociation rates vs the amount of off-target DNA we know that this rate does not make a difference in the presence of stripping, figure 4.2.4 (C). When IκBα is not actively removing NF-κB from the DNA site will the longer half-life of the off-target DNA result in an earlier onset of prolonged target DNA complex half-lives in the presence of small amounts of off-target DNA, compare to figure 4.2.4 (A). The effect on the full NF-κB response can also be achieved by lowering the NF-κB:IκBα association rate 10 fold but we have here chosen to change the dissociation rate from the off-target DNA as this rate is already an assumption based on the different observations reported in the literature. It might, however, be relevant to change the association rate of the transcription factor and the inhibitor protein as this rate is only an estimation in the previous models.

Elevated basal nuclear NF-κB level.

Including DNA interaction in the full NF-κB model results in elevated basal levels of nuclear NF-κB, see figure 4.2.5 (B). Biochemical experiments as EMSA and single cell fluorescent imaging exhibit no NF-κB in the nucleus before stimulation [Hoffmann et al., 2002, Werner et al., 2008, Nelson et al., 2004, Lee et al., 2009], indicating that there is no NF-κB bound to the DNA in the basal state. The basal nuclear NF-κB level is highly elevated in simulations including DNA interaction, even when no off-target DNA is present. The transcription factor binds the DNA and the slow off-rate traps NF-κB in the nucleus. Introducing stripping by the inhibitor protein actively removing the TF from the DNA brings down the basal level of DNA bound NF-κB more than 50%.

Off-target DNA

A more realistic model of the biological system is having large amounts of off-target DNA to which the TF can bind shortly but not resulting in gene transcription. Large amounts of off-target DNA in the simulations slows down the dynamics of the tran-
Figure 4.2.5: A: Schematics of the DNA module implemented into the full Werner 2008 NF-κB model. B: Top: full model simulations with the DNA interaction module reveals an important role of IκBα mediated dissociation of the NF-κB in bringing down the basal nuclear NF-κB level. This effect is even more pronounced in the presence of large amounts of off-target DNA, blue bars. C: full model simulations including DNA interactions with 20nM target DNA and no off-target DNA (top) and with 2μM off-target DNA. The measure of nuclear NF-κB is the sum of all NF-κB bound to both target and off-target DNA and the IκB:NF-κB:DNA complex of both DNA types, as this is the measured levels by EMSA in [Hoffmann et al., 2002].
system within the experimentally measured rates. We have in (paper 1) revealed the ability of IκBα to actively dissociate the NF-κB:DNA complex. This finding is novel and very important for understanding the dynamics of the transcription factor.

Here we present a simple kinetic model of transcription factor-DNA interaction and we are using it to analyze the effect of stripping in a system of more DNA types with different affinities for NF-κB. We propose that stripping not only maintains the fast dynamics of the transcription factor but additionally makes the system robust to changes in the level of off-target DNA sites in the nucleus and to the transcription factor affinity for the target DNA. In more physiological terms this is interesting, as different cells have different levels of DNA accessible during the cell cycle, cell division and also between cell types. Despite large variability in the DNA levels from cell to cell, the dynamics of the NF-κB•DNA complex can still be kept the same as long as the inhibitor protein is stripping the transcription factor off the DNA. It is in the same way possible that the transcription factor will interact with many genes of different affinity, but the overall transcription factor-DNA dynamics is held the same by stripping.

When including the DNA interaction module in the full NF-κB model earlier presented by [Werner et al., 2008] stripping is found not only to be responsible for fast NF-κB nuclear activity but to affect the basal level of the TF•DNA complex and thus the resetting of the system. Experimentally it has been shown how there is no free nor bound NF-κB in the nucleus in the resting cell. The trapping of the TF by the inhibitor proteins in the cytoplasm inhibits the nuclear TF activity, but most of all it allows the system to be reactivated. If there is still some TF bound to the DNA long after activation the stimulus specific gene regulations will be less efficient, as some gene transcription will potentially always be active.

The typology of having a protein actively disrupting the activity of a transcription factor by removing it from the DNA site is most likely not exclusive for the NF-κB signaling pathway (e.g. Systems of inducible activator). However, we do still not know how the stimulus specific gene expression is being preformed and how and if the temporal profile of the nuclear NF-κB activity plays a role in gene expression.
Patterns and Mutations in Tissue Growth

We are now leaving the gene regulatory networks and protein - DNA interaction considerations and moving up one level to the interaction between cells. The following chapter represents a study of tissue growth and mutations, where we consider a tissue spanned by a 2D network of cells placed on a hexagonal lattice, fig. 2.2.2. We build tissues by different growth techniques and analyze the effect of mutations in these tissues.


5.1 Introduction

During early development cells divide and eventually form organs and tissues which together build up the entire organism. While organs are three dimensional structures tissue is often close to two-dimensional and can be approximated by a single layer of cells. Tissues are formed through repeated cell division, where neighboring cells enter different developmental pathways, resulting in a fine-grained pattern of different cell states [Greenwald, 1992]. The most common mechanism that generates such patterns is lateral inhibition through Delta-Notch coupling [Sprinzak et al., 2010] or gap junctions, as described in section 2.2 on page 16.

Some tissues are in nature found to have hexagonal structures, as onion epithelium or human colon tissue, figure 2.2.1 on page 16. Here we simulate four different types of tissue growth, placing cells with bi-directed cell-cell interaction on a hexagonal lattice each interacting with its 6 neighbors through lateral inhibition. This type of idealized tissue is shown in figure 5.1.1.

Previously [Jensen et al., 2009] has presented a model of repressing genes on a hexagonal lattice such that gene is repressed by 3 out of the 6 neighboring genes. Under these conditions sustained oscillations in gene activity are found with only three allowed phases, each differing by $2\pi/3$.

Here we propose a model of cells placed on a hexagonal lattice with bi-directed interaction. We are likewise considering the activity of a certain gene/protein in the cell,
but the network is now spanned by the actual cells. The main difference is that proteins are usually free to move around within the cell whereas we can imagine structures of cells placed in a tight configurations, as in a tissue. The bi-directed interaction between the cells do not allow for oscillating states but a more switch-like behavior is seen where cells are either active or silenced. Each cell (x) is labeled by its coordinates in the tissue (m, n), and the dynamical equations of the protein activity of each cell is given by the below equation, as in [Jensen et al., 2009]:

$$\frac{dx_{m,n}}{dt} = c - \gamma x_{m,n} + \alpha F_{int}$$  \hspace{1cm} (5.1.1)

where $c$ is constitutive protein production, $\gamma$ is the protein degradation rate and $\alpha$ is the strength of the repression from neighboring cells. Further, $K$ is the dissociation constant of the binding complex and $h$ is the Hill coefficient, measuring the cooperativity of the binding [Jensen et al., 2009]. Cell-cell interaction happens through the term $F_{int}$ where repression is modeled with the use of standard Michaelis-Menten kinetics. The repression from the neighboring cells can be either additive:

$$F_{int} = \frac{1}{1 + \left(\frac{x_{m+1,n}}{K}\right)^h} + \frac{1}{1 + \left(\frac{x_{m-1,n+1}}{K}\right)^h} + \frac{1}{1 + \left(\frac{x_{m,n-1}}{K}\right)^h} + \frac{1}{1 + \left(\frac{x_{m-1,n}}{K}\right)^h} + \frac{1}{1 + \left(\frac{x_{m,n+1}}{K}\right)^h} + \frac{1}{1 + \left(\frac{x_{m+1,n+1}}{K}\right)^h}$$

or multiplicative:

$$F_{int} = \frac{1}{1 + \left(\frac{x_{m+1,n}}{K}\right)^h} \cdot \frac{1}{1 + \left(\frac{x_{m-1,n+1}}{K}\right)^h} \cdot \frac{1}{1 + \left(\frac{x_{m,n-1}}{K}\right)^h} \cdot \frac{1}{1 + \left(\frac{x_{m,n+1}}{K}\right)^h} \cdot \frac{1}{1 + \left(\frac{x_{m+1,n+1}}{K}\right)^h}$$

The below simulations are all performed with multiplicative repression and absorbing boundaries. The parameters used are $c = 0.1$, $\gamma = 1$, $K = 1$, $h = 2$ and $\alpha = 3$. 

Figure 5.1.1: A small part of a larger tissue spanned by cells on a hexagonal lattice. Cells are interacting through bi-directional repression.
5.2 Results

First we build a two-dimensional cell tissues through four different growth mechanisms: by cell division, cell migration, lateral growth, figure 5.2.1, or by preset random initial conditions.

**Cell Division**

Growing a tissue by cell division is the first type of growth I will present. Characteristic for cell division is that the new cell arising from the division is identical to its mother cell. We start with 7 cells at random initial conditions. When the protein levels have equilibrated and the cells are each in an individual state we let the top cells divide upwards and the two lower cells divide downwards, and the new cells are identical to the ‘mother’ cell, figure 5.2.1 (A). When the protein levels of this new configuration of cells are equilibrated we let the left most column of cells divide to the left and the right most column to the right, letting the system equilibrate before dividing the cells at the top and bottom again. This we continue till a reasonable sized lattice.

The final, equilibrated tissue has large areas of stable patterns, where one active (high) cell is surrounded by silenced (low) cells. A defect in the stable pattern has, however, arisen at the center of the tissue and does not disappear at further tissue growth, figure 5.2.5 (A). The defect at the center is fertilized already when the tissue only spans 4x4 cells, and is quantified as three silenced cells in a row. The defect stems from the way we build the tissue where we let more cells divide at once. Building the tissue by single cell division will lead to a completely ordered pattern of cell states across the tissue (results not shown here).
CHAPTER 5. PATTERNS AND MUTATIONS IN TISSUE GROWTH

Cell Migration

Next we imagine building a tissue where the existing cells are not dividing but the tissue is extended by migration of new cells from other parts of the organism. The state of the new cells is independent of the tissue itself, and we are here considering them all to be in an initial silenced state. Once the cells are joined to the tissue the protein levels will equilibrate and a new pattern is found. Building a tissue this way leads to a completely ordered pattern, where all active cells are surrounded by silenced cells.

Lateral Growth

The third kind of tissue growth is lateral growth where a new layer of cells build on top of existing layers of cells, in two dimensions. This illustrates the reproduced of our skin. We simulate lateral growth by only letting the top layer of the tissue divide, which adds a new layer of cells to the tissue in the same state as the cell of the top layer, figure 5.2.1 (C). The final tissue has large patches of ordered patterns separated by defects and unstable domain walls 5.2.5 (G).

Pre-set Tissue with Random Initial Conditions

In the fourth way of building the tissue we do not grow the tissue from a small number of cells but we define the tissue size and assign all the cells random initial protein conditions. When equilibrated, this tissue becomes very disordered with areas of different kinds of ordered patterns separated by unstable regions.

Interestingly, cell division and migration always result in the same tissue pattern when run multiple times, whereas tissues build by lateral growth or preset random initial conditions give very variable patterns.

The Stable Pattern

Before moving into the analysis of the different tissues and their robustness to mutations I will briefly touch upon the stable pattern of the cell activity in the hexagonal tissue. The stable pattern of cell states of a tissue with multiplicative bi-directed cell-cell interaction is the condition where all active cells are surrounded by silenced cells and where a silenced cell will have 3 active and 3 silenced cells surrounding it, figure 5.2.2 (A). This state is very robust and we will later see that it is difficult for a mutation in a single cell to have an effect on other cells in this kind of ordered state. The stable pattern is a consequence of geometry and is independent of the chosen parameters.

Figure 5.2.2: Two kinds of ordered patterns formed in a tissue spanned by multiplicative bi-directional cell-cell interaction on a hexagonal lattice.
5.2. RESULTS

Other kinds of stable ordered patterns can be observed, as the case where the pattern is formed of one row of silenced and active cells right next to each other, and one complete row above and below of only silenced cells, figure 5.2.2 (B). All active cells are in this way still surrounded by silenced cells but the silenced cells only have 2 active and 4 silenced cells as neighbors.

![Figure 5.2.3: Center part of a 50x50 tissue where cell division has been performed at random sites in the tissue. The chosen cell divides and subsequently pushes the other cells one unit over. Due to the symmetry of the hexagonal lattice the cell can divide in 6 directions, chosen at random. The remaining cell at the edge is neglected and simulations continue without.](image)

It is additionally possible to have different ordered patches in the same tissue, separated by lines or unordered domain walls. These domain walls are kept due to the rigid and stable surrounding pattern but are on their own not stable and tend to move around in the tissue as it is being grown (lateral growth). When dividing individual cells in a pre-set initial tissue, giving the new cell the same state as the mother cell and pushing all cells in one of the 6 directions of the lattice, we find that the pattern will slowly become more ordered. If, however, large areas of ordered patterns arise, but these are not compatible, random division of cells is not able to get rid of the frustrated lines in the pattern, between the ordered areas. In figure 5.2.3 we perform 30,000 divisions of cells chosen at random in a tissue originally made from a preset 50x50 tissue
with random initial conditions which has large patterns instabilities. Division of cells in the tissue slowly enhances the orderedness of the pattern but is not able to get rid of the remaining unstable lines. Specific cell division at the frustrated boundary will potentially resolve parts of the unstable area which random cell division can not achieve.

All simulations have been performed with multiplicative cell-cell interactions. The stable pattern which arise in a tissue built of cells subject to additive repression is found to be different from and even opposite to the one discussed above. This I will no elaborate further on here.

How Mutations Effect Cells in the Tissue

Figure 5.2.4: Quantization of the effect of a mutation of a single cell in the tissue. A mutation is performed by switching the activity of a single node and keeping it fixed for the remaining simulation time. We record the total change in the activity of th cells in the tissue due to the mutation and the number of cells affected by switching a particular node activity.

We have mutated individual cells in the tissue to investigate the stability of the different patterns. Mutations are performed by switching the activity of a single cell from active to silenced or from silenced to active, and keeping it fixed for the remaining simulation time. We record the total change in the protein activities of all the cells in the tissue, due to the mutation. In addition we count the number of cells which have a change in activity of more than 5% of the maximum protein activity of the cell, figure 5.2.4.

Mutations have been performed for every single cell in the tissue and the results are summarized in figure 5.2.5. The top 4 panels of the figure are the initial tissue, built by the four different techniques described above. The middle panel displays the result of mutations, where the color of individual cells now represents the total effect on the protein levels of the cells in the tissues above, caused by a mutation in this cell. Likewise, the color of the cells in the tissues in the lower panel represents the count of cells affected by the mutation in the specific cell, according to the color bar on the left. The total effect of the mutation on the states is found by taking the sum of the absolute change in protein activity of all cells in the tissue. In this way cells which are strongly effected by the mutation are higher represented in the measure than cells which state is only slightly affected. As we are measuring the absolute change is this independent on whether there is a decrease or increase in cell activity.

We have for the tissues in figure 5.2.5 drawn lines at the boundaries between the ordered and disordered pattern areas under the assumption that the ordered area has two silenced cells between every active cell, and all active cells are surrounded by silenced cells, figure 5.2.2 (A). The drawn lines makes it easier to see the differences in
5.2. RESULTS

Starting with the completely ordered pattern of the tissue built by cell migration, figure 5.2.5 (A, E, I), we see that the effect of a mutation follows the cell activity pattern of the original tissue. Only cells at the top left and the lower right corner have a little larger effect on the surrounding cells when mutated. This is because they are at the corners of the tissue and the protein levels of these cells are not stabilized from all sides, as cell located in the middle of the tissue are. When looking at the lower panel of the count of cells affected by the mutation, we see that mutations are more likely to have an effect, not only at the corners but at the entire boundary of the tissue. This is a general phenomenon and is also seen at the outermost boundary of the ordered parts of the tissue built by division, figure 5.2.5 (B, F, J) and to the right in (K) of lateral growth.

Just by looking at the results of the four mutated tissues summarized in figure 5.2.5 we notice that silenced cells (initially white cells in the tissue) mutated active has a larger effect on the state of the cells in the tissue than then mutating an active cell silenced (light blue color bar is a higher value than dark blue). Additional we see that large effects of the mutations predominantly happens at the boundary between the frustrated and ordered patterns. This I will elaborate on in the following sections.
Silenced Cells Mutated Active has Larger Affect than Silencing Active Cells

The effect of mutating a silenced cell to active is larger than when silencing an active cell. A silenced cell is surrounded by 3 other silenced cells and 3 active cells in the ordered pattern formation, and an active cell is surrounded by only silenced cells. Making a silenced cell active will destabilize the other active cells close by, causing a rearrangement of the active and the silenced cells in the surroundings. When mutating and keeping an active cell silenced, the repression on the neighboring cells will be released and allow for a slight increase in protein activity. This latter effect is not as fatal for the pattern structure as when introducing another active cell in the stabilized state pattern.

Table 5.2.1 provides statistics of the effect of mutations in the four different tissues, measured as the count of cells which activity is affected more than 5% (the same measurement as in the lower panels of figure 5.2.5). We are here limiting the analysis to contain only cells which are 3 cells from the boundary of the tissue, in order not to include the boundary effect discussed above. If we again start with the ordered pattern in the tissue built by migration we find that both in an 18x18 cell tissue and in the larger 50x50 cell tissue the active cells mutated silenced will not have any countable effect on the pattern (mean: 0, std: 0), whereas the silenced cells mutated active, affect 3 cells (mean: 3, std: 0).

Figure 5.2.6: The effect of mutations when active cells are mutated silent and silenced cells are mutated active in a tissue spanned by 18x18 cells. In the two lower panels the top distributions have been split out into the two groups of silenced cells and active cells before the mutation. Within these groups are the cells which are located within 2 cells of a defect colored green/red and the cells located 2 or more cells from a defect colored blue/yellow. The mean and standard deviation can be found in table 5.2.1.

Figure 5.2.6 (top row) display the distributions of number of cells which cause a certain count of cells affected more than 5% by the mutation. The cells are split into
5.2. RESULTS

<table>
<thead>
<tr>
<th>Tissue growth (18x18):</th>
<th>Cell div.</th>
<th>Migration</th>
<th>Lateral</th>
<th>Random</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silenced to active, mean (std)</td>
<td>5 (2)</td>
<td>3 (0)</td>
<td>4.6 (2)</td>
<td>6.7 (3.2)</td>
</tr>
<tr>
<td>Active to silenced, mean (std)</td>
<td>1 (1.3)</td>
<td>0 (0)</td>
<td>1.0 (1.4)</td>
<td>3.3 (2)</td>
</tr>
</tbody>
</table>

within 2 cells of a defect

| Silenced to active, mean (std) | 5.8 (2.1) | - | 5.6 (2.3) | 6.7 (3.2) |
| Active to silenced, mean (std) | 1.6 (1.4) | - | 1.7 (1.5) | 3.3 (2) |

2 or more cells from a defect

| Silenced to active, mean (std) | 3.5 (0.9) | - | 3.5 (1) | 6.5 (5) |
| Active to silenced, mean (std) | 0.25 (0.7) | - | 0.1 (0.4) | 2 (0) |

<table>
<thead>
<tr>
<th>Tissue growth(50x50):</th>
<th>Cell div.</th>
<th>Migration</th>
<th>Lateral</th>
<th>Random</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silenced to active, mean (std)</td>
<td>3.5 (1.2)</td>
<td>3 (0)</td>
<td>4.4 (2)</td>
<td>6.7 (2.8)</td>
</tr>
<tr>
<td>Active to silenced, mean (std)</td>
<td>0.3 (0.8)</td>
<td>0 (0)</td>
<td>1.0 (1.4)</td>
<td>2.8 (2)</td>
</tr>
</tbody>
</table>

within 2 cells of a defect

| Silenced to active, mean (std) | 5.3 (1.5) | - | 5.3 (2.2) | 6.8 (2.8) |
| Active to silenced, mean (std) | 1.7 (1.3) | - | 1.8 (1.5) | 3.1 (1.9) |

2 or more cells from a defect

| Silenced to active, mean (std) | 3.2 (0.7) | - | 3.2 (0.8) | 5.7 (2.0) |
| Active to silenced, mean (std) | 0.04 (0.3) | - | 0.06 (0.3) | 0.7 (1.1) |

Table 5.2.1: Mean number of cells affected (more than 5%) of a mutation of either a silenced cell to active, or an active cell to silenced. The cells are grouped according to their position relative to a defect in the tissue. Only cells which are at a distance of 3 cells or more from the boundary are included of the 18x18 tissue (top) and the 50x50 tissue (bottom) to avoid boundary effects. A tissue built by migration has no defects and no numbers in the 4 lower rows. The average also includes the cells, which when mutated do not have any effect above 5%, thereby contributing by zero and lowering the average).

Two distributions, one of the cells initially active and mutated silenced (blue), and those which were silent and mutated active (red). The effect of the mutation on the pattern in the tissue built by division, lateral growth and random initial conditions is different from that of the completely ordered pattern in the tissue built by migration, as the three tissues include areas of instability which affects the effect of the mutation. We can conclude that a mutation has a larger effect on the pattern when mutating silenced cells active than when silencing active cells.

The activity of the cells in a tissue will always try to reach the stable pattern and can use mutations in frustrated areas to gain more order in this area. This we have analyzed by dividing single cells in the tissue and letting the new cell push the existing cells to the side, as discussed on page 50. This creates a huge instability in the direction of division. This instability will always be corrected if it happens within a pattern of ordered states, as the pattern on both side of the frustration line is ordered and rigid, pushing the frustrated cells back into order. It is, however, hard to correct an instability if the ordered patterns on each side are not synchronized, figure 5.2.3.

Mutations at the Boundary of a Frustrated Area has Larger Effects

Additionally, when looking at the effect of mutation on the cell activity in figure 5.2.5 we observe that the mutations performed close to the boundary of an unstable area tend to have larger effects. We analyze this by recording both the rate of the cell before
mutations and the location of the cell relative to a frustrated area.

In the lower two rows of figure 5.2.6 the cell are further split into two groups, those which are within 2 cells of an unstable boundary (green for active cells and red for silenced cells) and those which are 2 cells or more from the boundary (blue for active cells and yellow for silenced cells). Active cells mutated silent are blue/green and silent cells mutated active are red/yellow. Although the distributions are not completely separated it is clear that cells located within 2 cell of the boundary cause a larger effect on the over-all cell activity in the tissue when mutated, compared to cells which lie away from the boundary. The mean and standard deviation of the distributions can be found in table 5.2.1. The statistics has been derived both from a tissue of 18x18 cells and of a larger tissue spanned by 50x50 cells.

Finally, in the aim of analyzing this effect of mutating cells at the boundary we have measured the number of cells which are part of an unstable pattern, within a radius of 1.5 cells from the references cell, see insert of figure 5.2.7. The count of cells which are affected by the mutation is plotted against this measure of the relative instability close to the cells, where the node size scale with the number of cells recorded having this location and causing the given effect when mutated. First of all we see that only active cells mutated silent have no effect when mutated, as earlier observed. The near presence of more cells being part of an unstable area tend to allow for a larger effect of a mutation of an active cell to silenced. The large but rare maximal spread from an active cells is seen with 8 instable cells within 1.5 cells radius of the cell, which affects 12 cells near by. The silent cells mutated active are causing a substantially larger effect when mutated. The largest effect is not seen when the cell is completely surrounded by unstable pattern but more in an area where the cell has 2-4 of these cells close by. The largest effect from the mutation of a silenced cell is recorded with 3 instable cells close to the cell which results in a mutation effect of 21. An area of relatively low mutation spread is shared between the active and silenced cells, figure 5.2.7.

5.3 Discussion

This study analyses the formation of different patterns during tissue growth and the effect of mutations in these tissues. We find that mutating silent cells active always has an effect on the surrounding cells. Mutating active cells silent has, however, only an effect when the cell is located near an unstable boundary. Close to the boundary all cells show an effect upon mutation, whereas within the tissue only silent cells show an effect.

The mutations only have an effect on the cells located near the cell of reference, but mutations does tend to affect cells in the unstable area seeking to gain a stable pattern of protein activity in the cells. The stable configuration functions as a ‘wall’ and the mutation can not permanently change the activity of cells in this stable pattern.

Future work would include further analysis of additive repression and the states and stability gained. Also we can vary the strength of the coupling between the cells, illustrating the inhomogeneous protein distribution of cells in the tissue.
Figure 5.2.7: Count of cells affected more than 5% of a mutation in a 50x50 tissue as a function of the number of cells part of an unstable area, within a radius of 1.5 cells from the references cell. Squared, red nodes are silenced cells mutated active and round, blue nodes are active cells mutated silenced. Node size scale with number of cells recorded to have this specific location and mutation effect.
Network Analysis of Human Interactions

Leaving the gene regulatory networks of NF-κB transcription factor activity and the cell-cell repression study I will now zoom yet another step out and look at interactions between human beings. This study is done in collaboration with Lilla Sipos, PhD student in psychology at the University of Budapest, Hungary, analyzing the interaction patterns between mothers and infants at the age of 1 year comparing full-term and preterm born infants.


6.1 Introduction

Preterm infants are more likely to experience a developmental delay than their full-term peers. Full-term infants are born at the end of the gestational period whereas the preterm infants are born at less than 37 weeks of gestation. The birth weight of preterm infants is less than 2500 grams, depending on the demographic, economic and medical conditions. Prematurity is not an illness and does not always cause a developmental delay, but preterm babies are at risk of lacking cognitive and social development.

The premature birth of a child requires a higher degree of adaptation from the mother and sets an atypical condition for the mother-infant interactions as the mother might not be ready to meet the needs of the child. Whereas research in this field has mainly focused on the risk factors associated with preterm birth, an increasing number of studies are now turning to the quality of the mother-infant interaction [Fiese et al., 2001, Klein and Feldman, 2007, Landry et al., 2000]. Reported data on the behavior of the preterm infants are inconsistent [Bakeman and Brown, 1980, Goldberg and DiVitto, 1995, Minde et al., 1985], which might be due to various factors as degree of immaturity, perinatal complications in the infant, preparedness of the mother, support available to the mother, the infant’s age and the context of the interaction analyzed.

Here we use a mutually exclusive and exhausting micro-analytical category system to characterize the mother-infant interactions at the age of one. We attempt to eliminate inconsistency by an in-depth analysis of the coded data through formation of complex interaction networks and transition patterns between combined infant/mother states.
We find that the preterm group shows a large diversity in the nature of mother-infant interactions and that the preterm interaction is often less harmonious.

Data Collection

30 preterm babies born between week 28-33 of gestation with birth weight of 800 - 1900 grams participated in the study, all recruited from a hospital in Budapest (Hungary). The babies had no congenital abnormalities or obvious sensory defects and were considered by the neonatologists as low- to moderate risk babies. The male/female ratio was 47/53%. These babies were compared to 42 full-term babies born in week 37 of gestation or later, with a mean body weight of 3421 grams and a male/female ratio of 52/48%. The mean age of the mothers was 28.3 years (range 20-42) in the preterm group and 26.6 years in the full-term group (range 19-34). Statistical tests performed by Lilla Sipos revealed that the two groups of mothers had matching demographic variables, only did the mothers of the full-term babies have a higher level of education (p<.005). The initial study follows the infants till the age of 6. We are, however, limiting our analysis to the mother-infant interaction when the infant is one year old, which has been decided by Lilla Sipos to be the time of more relevance for our study.

The mothers and infants were video recorded in a play situation at home, recorded by a female researcher. The visit always began with a 10 min familiarization period, followed by 5-10 min where the mother was asked to play with her child as she ordinarily would. Lilla Sipos has developed a scheme of 8 different child behaviors ("state") and 11 different mother behaviors (table 6.1) and used this in the characterization of the interaction. She has for every second in the recorded mother-infant interactions denoted a behavioral state to each of the child and the mother. Micro-analytic coding systems have already been developed and used by [Bakeman and Brown, 1986], but the scheme is not as extensive and with the time resolution obtained by Lilla Sipos. More detailed description of the individual categories can be found in Appendix A.

<table>
<thead>
<tr>
<th>Mothers</th>
<th>Children</th>
</tr>
</thead>
<tbody>
<tr>
<td>(10 - other)</td>
<td></td>
</tr>
<tr>
<td>11 - follow</td>
<td>1 - plays</td>
</tr>
<tr>
<td>12 - enriches</td>
<td>2 - explores</td>
</tr>
<tr>
<td>13 - physically forces</td>
<td>3 - obeys</td>
</tr>
<tr>
<td>14 - commands</td>
<td>4 - cooperates</td>
</tr>
<tr>
<td>15 - direct attention</td>
<td>5 - disobey</td>
</tr>
<tr>
<td>16 - interrupts</td>
<td>6 - neglects</td>
</tr>
<tr>
<td>17 - passive</td>
<td>7 - passive</td>
</tr>
<tr>
<td>18 - neglects</td>
<td>(8 - other)</td>
</tr>
<tr>
<td>19 - insensitive</td>
<td></td>
</tr>
<tr>
<td>20 - searches/handles toy</td>
<td></td>
</tr>
</tbody>
</table>

Table 6.1.1: Micro-analytic categories for the behaviors of the mothers and the infants, respectively. The analyses have all been performed without the categories ‘other’ as these seldom happen and are not quantitative for the interaction between the mother and the infant.

¹Doctor specialized in medical care of newborn infants
6.2 Results

Network Analysis of Interaction data

The first analysis we perform is to look at the change in behavior of the mothers and of the infants, separately, during the recorded play-session. The time series data has been mapped as a network using Cytoscape [Shannon et al., 2003] where each node represents an infant or maternal state and the links between the nodes represent a change in behavior. Time is neglected in the network representation and one can not tell in which order the transitions occurs (we will later analyze the progression of the transitions in time). The width of the links scale with the transition rate, given by how many times this specific change in behavior occurs, normalized to the total number of changes in behavior in the entire group of either preterm or full-term mothers/infants. The transition rate is in this way normalized to both the number of cases in each group and the variability in the recording length. The size of the nodes scale with the average time spend in the particular state and the arrow heads indicate the direction of the change in behavior.

To highlight the differences in the behavioral patterns of the two groups of preterm and full-term infants we have subtracted the normalized behavioral networks.

![Network Analysis of Interaction data](image)

Figure 6.2.1: Difference between full-term and preterm interaction networks of the mothers (left) and infants (right) separately. Each node represents an infant or maternal behavior ("state"), numbers referring to table 6.1. The size of the nodes scale with the difference between the average time spent in the state, dark grey where preterm spend more time and white where full-term do so. The links between the nodes represent transitions between states, and the width of the link scales with the difference between the normalized transition fractions in the two groups. Red arrows are the transitions which occur more in full-term and blue arrow are transitions which occur more in preterm interactions. We have applied a threshold of 1 in the difference between the two groups, below which the arrows are light grey. Arrow heads indicate the direction of the change in behavior.

Isolated Infant Behaviors

Subtracting the behavioral networks of the infants recorded in a play situation with their mothers show a few very distinct full-term transitions in the right part of the network plot in figure 6.2 (right), occupying the states of 1-4, which account for play (1), explores (2), obey (3) and cooperates (4). Only one distinct preterm transition from dis-
obeys (5) to explores (2) appears. In general, only weak distinct preterm transitions are seen, compared to full-term transitions, indicating that the preterm infants are more diverse in their interaction pattern. The size of the node scales with the difference in average time spent in the given state. Full-term stays on average 35% longer in play (1) (mean (std): Full-term = 28.4 (36.7) sec, preterm = 21.0 (29.2) sec) and remain 45% longer passive (7), than preterm. The distribution of times spent in play are compatible between the two groups, but where full-term stay up to 354 sec in play is the longest time observed in play for the preterm 251 sec. Comparing the difference between the two groups to the standard deviation of the time which full-term infants spent in play, with the present sample size of all transitions recorded is the difference statistically significant [Whitley and Ball, 2002] (p<0.05).

Preterm infants only stay slightly longer in the two behavioral states of obeys (3) and disobeys (5), compared to their full-term peers. The mean and standard deviation for the time spent in all 7 infants states are listed in Appendix A.

Isolated Mother Behaviors

The difference in the behavioral networks of the mothers also shows a clear tendency that the mothers of full-term born infants have certain strong behavioral transitions compared to the mothers of the preterm infants. This is between the behavioral states of follows (11), enriches (12) and direct attention (15). The distinct behavioral transitions of the mothers of the preterm infants lie between direct attention (15) and physically forces (13) and between direct attention (15) and neglects (18), where the mothers often switch between pushing her own ideas (direct attention) to either using physical force or neglecting. Compared to full-terms, mothers of preterms seem to insist on their ideas more intensively, and accept the infant's different wishes less often. The mothers of preterms are additionally slightly distinct in a number of behavioral transitions spanning a larger part of the network indicating that the mother of the preterm infants are in many of possible states. As time is lost in this network representation of the data we can not specify the sequence of distinct transitions, but it looks as if there is a possible cycling between the two mother states for the mothers of the full-term infants of follows (11) and enriches (12). This will be analyzed in further detail on page 66.

These results suggest that interaction of full-term infants and their mothers are more focused and harmonious, and full-term infants transitions are more frequent between playing, cooperating, exploring and obeying. The major difference in the maternal behaviors is that mothers of full-term infants alternate between following and enriching the infants activity and directing his/her attention to new ideas more often than mothers of preterm infants. Our results also show that mothers of preterms more often switch from pushing their ideas (direct attention) to intrusive (using physical force, 15\leftrightarrow13) or neglecting (15\leftrightarrow18) behaviors.

Combined Mother-Infant Interaction

Already now we have an idea that the preterm infant-mother interaction is less harmonious compared to their full-term peers. Next we will look at the combined behavioral networks where each state is a joined behavior between the mother and infant and thus represents their interaction at a given time point.

The first analysis concerns which of the 70 possible combined states are visited by full-term compared to the preterm. We find that preterm are in all but one state com-
Figure 6.2.2: Top: Interaction network of the combined mother-infant behavioral transitions in full- and preterm. Nodes represent a combined mother-infant behavioral state and size scales with the average time spent in the state, given by the child state in the outer ring, combined with the mother states, running from 11 to 20 within each section of a child state. The corresponding behavior of the mother is found in the list on page 60. The links between the nodes are the transition from one mother-infant state to the other, and the width and color scale with the transition rate, normalized within the group of either full-term or preterm infants. A threshold of 1 has been set, below which transitions are considered random and are colored green (see power density plot, figure 6.2.4). Bottom: Subtractions of the behavioral networks of the full-term and the preterm mother-infant interactions. Red arrows are the full-term behavioral transitions which have a difference of 0.5 or more to the preterm and blue arrow are preterm transitions which have a difference in transition rate of 0.5 or more than in the full-term peers. The width of the links scales with the difference in transition rate and the node size scale with the difference in time spent in the state.

bination; cooperates (4) - searches toy (20). The full-term are on the other hand leaving out the following 8 combinations of states:

This shows how the preterm are in more of the possible states, and that the states where they differ from the full-term are associated with the more negative behaviors, as disobey, interrupts, passive, insensitive and neglects.
neglects (6) - interrupts (16)  
obey (3) - passive (17)  
disobey (5) - passive (17)  
passive (7) - passive (17)  
disobey (5) - neglects (18)  
disobey (5) - insensitive (19)  
passive (7) - insensitive (19)  
passive (7) - searches toy (20)

Next, we visualize the behavioral patterns as a network, where the nodes now represent a combined mother-infant behavioral state. The size of the nodes scale with the average time spent in the combined state and the name of the state is given by the child state in the outer ring, combined with the mother states, running from 11 to 20 within each child state. The corresponding maternal behavior can be found in the list on page 60. The color and width of the links scale with the transition rate, normalized within the group of either full-term or preterm, figure 6.2.2. We have set a threshold of 1 below which the transitions are considered random. This will be discussed in further detail in section 'Statistical Strength' below.

To be able to tell if there is a difference between the full-term and preterm interaction patterns, the behavioral networks of the full-term and the preterm mother-infant interactions have been subtracted and any difference above 0.5 is displayed in the lower panel of figure 6.2.2. Red arrows are the transitions where the full-term are distinct and blue arrows are transitions distinct for the preterm. Node size scales with the difference in average time spent in a state. Full-term mother-infant interactions stay on average longer in one state, as seen by the positive full-term difference in the node size. Preterm spend slightly longer in cooperates (4)-direct attention (15) and explores (2)-follows (11).

Distinct full-term transitions are seen between the states of follows (11) and enriches (12) while the child stays in play (1). This is defined as harmonious mother-infant interaction and is interestingly very distinct in the full-term interaction (p<0.005). The interaction is only harmonious if the mother is in either state 11 or 12 while the infant is playing. If the child is playing but the mother is doing other things the interaction is not successful (reference: Lilla Sipos).

Coarse-Grained Degree Distribution

![Coarse-grained degree distribution](image)

Figure 6.2.3: Coarse-grained degree distribution of the combined mother/infant network.

A coarse-grained degree distribution analysis has been performed on the data, separating the combined behavioral states into the 5 categories listed to the right in fi-
The 5 categories separate the states into the very sparsely connected nodes which have either one input and one output (1) or either no input or no output (5), states which have many links leading into them, but only has one output (2), states which spread information in the network, having only one input but many outputs (3) and finally those who have many inputs and many outputs (4). We see that the distributions are similar, but there is a clear differences between full-term and preterm as full-term have a more diverse distribution of connectivity. The preterm interactions are highly connected supporting the hypothesis of the preterm being less focused and more diverse in their interaction with the mother.

Before proceeding into the discussion of potential transition paths I will briefly touch upon the statistical analysis of data.

**Statistical Strength**

We have in the above described network analysis used 1 as a cutoff for the significant non-subtracted transitions. The transition rates are small and lie between 0 and 5% because they are normalized to the total number of transitions in the system (full-term/preterm: 1728/1863). The distribution of the transition rates does, however, show a natural cutoff at 1.

![Figure 6.2.4: Distribution of the transition rates in full-term and preterm combined behavioral networks. The transition rates are measured as the normalized percentage within each group. The probability density function of transition rates are plotted on double-logarithmic scales indicating an underlying scale free network with scaling exponent -2. The full drawn curve shows an associated random Poisson distribution derived around the mean of the transitions rates from both data sets, (mean= 0.15 ). The shaded area represents the cutoff at 1, above which we consider the events to be non-random. The insert shows the distribution of transitions rates on linear scale.](image)

If all transitions were equally likely would the transition probability of one transition be defined as $\frac{1}{\text{total possible transitions}}$, which in the full-term interaction network is equal to 0.22 (69 possible states) and in the preterm network is equal to 0.16 (62 possible states), both slightly higher than the mean of the distributions.

The probability density function of the link weights in the combined interaction networks is plotted on double-logarithmic scales indicating an underlying scale free network with scaling exponent -2, figure 6.2.4. The full drawn curve shows an associated random Poisson distribution derived around the mean of the transitions rates.
from both data sets, indicating that a group of the transitions are far above random. The shaded area mark the region of transitions rates above 1, which we considered significant. The distribution on linear axis can be seen in the insert of the figure.

### Randomized Network

The statistical significance of the most distinct transition can additionally be tested by generating random interaction networks to see if these random networks will have distinct transition between the same states as found in the interaction data.

We generate 5000 random networks of both full-term and preterm interactions. Each random network is generated by swapping two links and keeping the weight with the link. In this way the number of nodes and links are preserved as well as the connectivity and only the typology of the network is changed. This is called the local rewiring algorithm [Maslov and Sneppen, 2002], figure 6.2.5.

Each random preterm networks is subtracted from a random full-term network and we record every time the transition rate of the transition from $1 \rightarrow 14$ to $1 \rightarrow 15$ is equal to or more than the transition rate observed in the data. This total number is divided by 5000 (the number of randomizations performed) to get the $p$-value. The $p$-value of the transition from $1 \rightarrow 11$ to $1 \rightarrow 12$ is <0.005 and is thus highly significant.

### Normalizing the Difference

When comparing the two interaction networks we subtract the transition rate of the full-term and preterm infants but do not normalize this difference. It might be more correct to normalize the difference, as a change from zero to a relative small transition rate might be of more interest than the same difference between already high transition rates. Several consideration has left us with the unnormalized difference. The main consideration concerns which variable we normalize with respect to. To normalize the difference by the transitions rate of full-term will result in infinities for transitions which does not appear in the full-term and thus have a transition rate of 0. Do we on the other hand normalize to the maximum transition rate of the two, (full-term - pre)/(max(full-term,preterm)) will the measure be relative but not comparable across the sample. We could additionally normalize to full-term/preterm.

### Interaction Paths

From the subtracted transition networks, potential distinctive paths can be detected in the system. Therefore we recorded the sequences of transitions before each distinctive
transition and calculated how frequently a given state appears at the specific position in the sequences obtained. In figure 6.2.6 we show the preceding states for three transitions: $1 \to 11$, $4 \to 15 \to 4 \to 11$, and $6 \to 18 \to 6 \to 15$.

Figure 6.2.6: states leading up to distinct transitions found in the subtracted interaction networks. The percentage of all states leading up to a given transition is given in the brackets. Two or more states listed right next to each other share the percentage in the bracket.

The $1 \to 11$ transition, which occurs in the full-term group more often than in the preterm group ($p<0.005$), and is often periodic. In this case the infant plays on his/her own while the mother alternates between following and enriching his/her activity. The mother thus gives the control to the infant in choosing what to play with, but stays involved in the interaction and helps maintain the infant’s attention by occasionally enriching, elaborating the infant’s ideas. This maternal behavior is favorable in four aspects: (1) helps the infant to develop focused attention by staying longer in a certain activity, (2) teaches the infant new ways of playing with one or another toy, therefore facilitates acquiring knowledge and skills, (3) allows the infant to experience that he is an able-to-act agent (4) provides mutual happiness and satisfaction in the interaction. Interestingly, during the sequence preceding the $1 \to 11$ transition mothers of full-term infants are predominantly in states $11$ (follow), $12$ (enriches), or $17$ (handles toy), while mothers of preterm infants often can be found in state $11$ (command), controlling the infant’s activity.

The $3 \to 15 \to 4 \to 15$ transition (the mother directs attention while the infant switches from obey to cooperate, i.e. plays according to the mother’s idea happily) is more than twice as frequent in the full-term group as in the preterm group. In this transition the infant turns the interaction more harmonious. The typically subsequent transition is that the mother stops directing the infant ($4 \to 15 \to 4 \to 11$) which occurs about twice more frequent in the full-term group.
Transitions between states 6·15 and 6·18 (the mother directs/neglects infant while the infant neglects the mother) happen very rarely in the full-term group (6·18 → 6·15 in case of 1 infant, and 6·15 → 6·18 in case of 3 infants), and a play situation was reached 8 to 15 seconds after the transition, leading to a harmonious state (1·11) eventually (mean=298, std=258). In the preterm group the 6·15 ↔ 6·18 transitions occurred more frequently ($\chi^2 < 0.05$). We found at least one transition in 30% of mother-preterm infant observations, and in 23% of the cases we observed more than one transition. In these transitions the mothers of preterms react to the neglecting behavior of the infant by directing the infant's attention or withdrawing them-self from the interaction (neglecting the infant), often alternating these behaviors. Interestingly, in the preterm group the 6·18 → 6·15 transition only led to harmonious play (1·11) within 2 minutes (117s) or longer (mean=248s, std=91s), and in 21% of the cases the interaction never turned harmonious after this transition. In case of the 6·15 → 6·18 transition we found only one case when harmonious play (1·11) was reached in a short time (10 seconds), which represents about 5% of the cases. Our data shows that in the preterm group the 6·15 → 6·18 transition is one of the most unsuccessful (5% success) maternal transitions from the 6·15 state. The different reaction observed in the two groups could be explained by the more mature nervous system of full-term infants, which may make them less sensitive to their mother's withdrawing from the interaction.

6.3 Discussion

Comparison of preterm and full-term dyads shows that preterm dyads are often less harmonious, which may result in a developmental lag. We find that mothers of preterm infants are periodically at two extremes of maladaptive behavior: they are either intrusive or disengaged. The pairing of an oversensitive infant with an intrusive/disengaged mother may place the infant at risk for later emotional, cognitive and behavioral disturbance.

Several reports [Madden, 200, Mayer et al., 1995, Muller-Nix et al., 2004, Wijnroks, 199] have suggested that mothers who gave birth prematurely feel guilty because of the shortened intrauterine period and they may try to compensate the infant for it. The more controlling and attention-directive behavior can be an attempt to re-mediate their self-image. The paradox is that the more the mother tries to force her own idea over the infant's idea, the fewer positive answers she gets from the infant because preterm infants neglect the mother's controlling behavior. Subsequently, the mother disengages, and a negative circulus vitiosus is established. Nevertheless, in the full-term group both partners stay involved and they share a mutual focus of attention almost all the time.

Our results highlight important differences in the interactions of the two groups. However, the preterm group shows a large diversity in the nature of mother-infant interactions. Therefore following the infants development in the longitudinal study as the one we have our data from, recordings of mother-infant interactions till the age of 6, may be able to reveal the long-term consequences of maladaptive maternal behavior.

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*group of two people*
Concluding Remarks

This thesis represents 3 years of work summarized under the notion of interaction patterns. The projects presented are all treating problems of interaction between different players, whether it is proteins inducing gene expression, cells forming tissues or human interaction between a mother and her child.

The main approach has been small scale modeling where my co-workers and I have studied the nuclear response of the transcription factor NF-κB and its interaction with the DNA, with the aim of gaining new insights to these two parts of the signaling pathway. Based on these studies we have shown how the oscillation period of the nuclear activity profile of NF-κB can be modified and fine-tuned by the two negative feedback regulators, IκBα and A20-like proteins. The inhibitor protein IκBα is additionally found to have a functional role in removing the transcription factor from the DNA.

We use the same model approach to study the mutually inhibitory interactions between cells in a tissue. We find that the patterns in cell activity formed through this lateral inhibition are essential for the robustness of the tissue to mutations.

The fourth project is a project on data analysis of interactions between mothers and infants in the setting of the preterm birth of a child. We study the patterns of interaction and found significant differences between the two groups of preterm and full-term infants, primarily in the maternal behavior.

Small scale modeling of biological systems, I believe, will make an even greater impact in future research in contributing to the understanding of such systems. Experimental techniques increasingly advance and we will soon be exposed to details of components and interactions we did not imagine only a few years ago. This data can improve our understanding and help gain the information needed to perform the modeling. However, the main experimental challenge is continuously to perform as real experiments as possible and at the same time extract information such as time series profiles of relevant proteins, interaction partners and rates.

One way to get pathway specific information useful for modeling is to observe how a pathway responds to a well defined input. This kind of analysis has been done on yeast (McClean, 2009) which is a favorable system as you can have the whole organism inside a Microfluidics chamber. The Microfluidic device enables you to directly control the input and monitor output protein levels with fluorescent reporters. In larger organism this is not possible. Experiments of this kind are performed in cell lines but the question remains how to really mimic the physiological condition creating the input stress on a cell which is part of a larger organism.
Mathematical modeling is increasingly often being used in the aim of understanding a complexity too high for the single researcher to comprehend and the small models have the advantage that they are simple and can easily be understood and interpret. I hope for and believe in an increasing integration between mathematical modeling and experimental research, providing the strong combination needed for solving the many still open questions of how essential biological processes function and are regulated.
Appendix - Network Analysis of Human Interactions

Micro-analytical Coding System

The two tables below contain the detailed descriptions of the micro-analytical coding system used to decode the interaction between mothers and infants.

<table>
<thead>
<tr>
<th>Child</th>
<th>behavior</th>
<th>details</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>play</td>
<td>plays with a toy of his interest</td>
</tr>
<tr>
<td>2</td>
<td>explores</td>
<td>searches for/approaches new toy</td>
</tr>
<tr>
<td>3</td>
<td>obeys</td>
<td>without sign of happiness plays what the mother wants, complies the mother's wishes</td>
</tr>
<tr>
<td>4</td>
<td>cooperates</td>
<td>happily accepts mother's idea</td>
</tr>
<tr>
<td>5</td>
<td>disobeys</td>
<td>actively opposes against the mother's idea/command</td>
</tr>
<tr>
<td>6</td>
<td>neglects</td>
<td>ignores mother or her ideas, does not do what mother wants but does not express it openly</td>
</tr>
<tr>
<td>7</td>
<td>passive</td>
<td>is not involved in any activity</td>
</tr>
<tr>
<td>8</td>
<td>other</td>
<td>any behavior which does not match with any of the categories, and behaviors, which are reactions to interruptions due to natural setting (e.g. infant goes out of the room)</td>
</tr>
</tbody>
</table>

Table A.1: Description of the assigned infant behavioral states
### APPENDIX A. APPENDIX - NETWORK ANALYSIS OF HUMAN INTERACTIONS

<table>
<thead>
<tr>
<th>Mother</th>
<th>behavior</th>
<th>details</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>other</td>
<td>any behavior which does not match with any of the categories, and behaviors, which are reactions to interruptions due to natural setting</td>
</tr>
<tr>
<td>11</td>
<td>follows</td>
<td>follows the infant's idea, she adapts herself to the child, they focus on the same thing, mother is emotionally involved</td>
</tr>
<tr>
<td>12</td>
<td>enriches</td>
<td>enriches the infant's play with her own idea, but does not change the toy/game, elaborates the infant's play, shows a new aspect how to use a toy</td>
</tr>
<tr>
<td>13</td>
<td>physically forces</td>
<td>physically forces the child to do or not to do something, or she is physically intrusive</td>
</tr>
<tr>
<td>14</td>
<td>commands</td>
<td>expects or demands the child to do something intrusively directs the infant's attention</td>
</tr>
<tr>
<td>15</td>
<td>direct attention</td>
<td>She insists on her own idea, despite of that the child would like to do something else, or she suggests a new toy/game, however the child is actively involved in something</td>
</tr>
<tr>
<td>16</td>
<td>interrupts</td>
<td>interrupts the infant's play activity with anything else</td>
</tr>
<tr>
<td>17</td>
<td>passive</td>
<td>does not do anything, and emotionally uninvolved</td>
</tr>
<tr>
<td>18</td>
<td>neglects</td>
<td>does not play with the child, and actively does something else (eg. initiates talk with the researcher)</td>
</tr>
<tr>
<td>19</td>
<td>insensitive</td>
<td>any kind of behavior, when the mother does not satisfy the infant's obvious need, or expresses negative emotion about the infant's behavior, or expresses developmentally unreachable expectation to the child</td>
</tr>
<tr>
<td>20</td>
<td>handling toy</td>
<td>does not play but manipulates a toy for the favor of the child (eg. assembles toy)</td>
</tr>
</tbody>
</table>

**Table A.2:** Description of the assigned maternal behavioral states
Numbers for the Separate Mother and Infant Interaction Networks

General Numbers

<table>
<thead>
<tr>
<th></th>
<th>Full term</th>
<th>Pre term</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean nr of changes pr time unit (sec)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Child</td>
<td>0.0695 (100%)</td>
<td>0.0949 (136.5%)</td>
</tr>
<tr>
<td>Mother</td>
<td>0.0648 (100%)</td>
<td>0.0773 (121.2%)</td>
</tr>
<tr>
<td><strong>Sum of nr of changes in all runs normalized to nr of runs in the data group</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Child</td>
<td>25.8095 (100%)</td>
<td>40.6333 (157.4%)</td>
</tr>
<tr>
<td>Mother</td>
<td>24.2381 (100%)</td>
<td>33.7333 (139.2%)</td>
</tr>
<tr>
<td><strong>un-normalized sum of changes in state, count</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Child</td>
<td>1084</td>
<td>1219</td>
</tr>
<tr>
<td>Mother</td>
<td>1018</td>
<td>1012</td>
</tr>
<tr>
<td>combined states</td>
<td>1745</td>
<td>1871</td>
</tr>
<tr>
<td><strong>Mean length of time series (runs), sec</strong></td>
<td>389.0952</td>
<td>441.9667</td>
</tr>
</tbody>
</table>

Table A.3: From the top going downwards: Mean number of changes the mother and the child preforms separately normalized to the length of the individual time series. Sum of the number of changes in all the runs in one data group, normalized to the number of runs in the data group. Total number of changes in the files, unnormalized. Mean length of the time series for the 2 data groups.

Time Spent in each Infant State

<table>
<thead>
<tr>
<th>Behavior</th>
<th>Full-term</th>
<th>Preterm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 - play</td>
<td>28.4 (36.7) max 352</td>
<td>21 (29.2) max 251</td>
</tr>
<tr>
<td>2 - explores</td>
<td>9.2 (8.9) max 58</td>
<td>9.3 (8.2) max 41</td>
</tr>
<tr>
<td>3 - obeys</td>
<td>9.6 (8.0) max 48</td>
<td>11.1 (11.5) max 108</td>
</tr>
<tr>
<td>4 - cooperates</td>
<td>20.1 (21.7) max 127</td>
<td>16.9 (16.6) max 116</td>
</tr>
<tr>
<td>5 - disobeys</td>
<td>4.2 (3.8) max 19</td>
<td>6.2 (5.5) max 25</td>
</tr>
<tr>
<td>6 - neglects</td>
<td>12.1 (12.6) max 97</td>
<td>10.8 (10) max 61</td>
</tr>
<tr>
<td>7 - passive</td>
<td>14.8 (13.7) max 65</td>
<td>10.23 (9) max 44</td>
</tr>
</tbody>
</table>

Table A.4: Mean (std) and maximum time in seconds the infants of the two groups are recorded in each of the 7 behavioral states.
Distinct Transitions in the Networks

<table>
<thead>
<tr>
<th></th>
<th>Infants</th>
<th>Full-term</th>
<th>Preterm</th>
<th>Difference</th>
<th>Fraction of max(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Full-term distinct transitions</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 → 4</td>
<td>6.2</td>
<td>2.98</td>
<td>3.22</td>
<td>3.22</td>
<td>52</td>
</tr>
<tr>
<td>4 → 2</td>
<td>5.73</td>
<td>3.55</td>
<td>2.17</td>
<td>2.17</td>
<td>38</td>
</tr>
<tr>
<td>3 → 1</td>
<td>4.23</td>
<td>2.15</td>
<td>2.08</td>
<td>2.08</td>
<td>49</td>
</tr>
<tr>
<td>1 → 3</td>
<td>4.79</td>
<td>2.73</td>
<td>2.06</td>
<td>2.06</td>
<td>43</td>
</tr>
<tr>
<td>4 → 1</td>
<td>3.94</td>
<td>2.07</td>
<td>1.88</td>
<td>1.88</td>
<td>48</td>
</tr>
<tr>
<td>1 → 2</td>
<td>6.39</td>
<td>4.88</td>
<td>1.51</td>
<td>1.51</td>
<td>24</td>
</tr>
<tr>
<td>1 → 4</td>
<td>3.79</td>
<td>2.48</td>
<td>1.28</td>
<td>1.28</td>
<td>34</td>
</tr>
<tr>
<td><strong>Preterm distinct transitions</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 → 2</td>
<td>0.66</td>
<td>2.07</td>
<td>1.41</td>
<td>1.41</td>
<td>68</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Mothers</th>
<th>Full-term</th>
<th>Preterm</th>
<th>Difference</th>
<th>Fraction of max(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Full-term distinct transitions</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11 → 12</td>
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<td>1.3</td>
<td>1.1</td>
<td>1.1</td>
<td>85</td>
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Table A.6: Transition rates of distinct infant transitions (above) and distinct maternal transitions (below), above a difference of 1.

The transitions found to have a difference between the full-term and preterm infants of 1, are listed in the below table. Going from left to right we have the transition is given in the far left column, followed by the transition rate is the full-term infants, the preterm infants and then the difference between the two. The fraction this difference represents, taken of the maximum of the two groups is displayed in the far right most column.

Numbers for the Combined Mother-Infant Interaction Networks

Like for the separate behavioral networks above, we are here displaying the distinct transitions, transition with a difference above 0.5, observed in the combined mother-
infant behavioral network.

### Distinct Transitions in the Networks

<table>
<thead>
<tr>
<th>Transition</th>
<th>Full-term</th>
<th>Preterm</th>
<th>Difference</th>
<th>Fraction of max(%)</th>
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<td>3→15 → 4→15</td>
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<td>0.07</td>
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<td>1.16</td>
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</tr>
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<td>1→11 → 2→11</td>
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<td>0.53</td>
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<td>1</td>
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<tr>
<td><strong>Preterm distinct transitions</strong></td>
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<tr>
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<td>1→18 → 1→11</td>
<td>0.12</td>
<td>0.64</td>
<td>0.53</td>
<td>82</td>
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</tbody>
</table>

**Table A.7**: Transition rates of distinct combined mother-infant transitions above a threshold of 0.5. The transitions are grouped so that the distinct full-term transitions are above, and the distinct preterm transitions.
Bibliography


Publications
Paper 1

Kinetic enhancement of NF-κB-DNA dissociation by IκBα

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Department of Chemistry and Biochemistry, University of California at San Diego, La Jolla, CA 92093-0378

Edited by Gregory A. Petsko, Brandeis University, Waltham, MA, and approved September 21, 2009 (received for review August 3, 2009)

A hallmark of the NF-κB transcription response to inflammatory cytokines is the remarkably rapid rate of robust activation and subsequent signal repression. Although the rapidity of postinduction repression is explained partly by the fact that the gene for IκBα is strongly induced by NF-κB, the newly synthesized IκBα still must enter the nucleus and compete for binding to NF-κB with the very large number of κB sites in the DNA. We present results from real-time binding kinetic experiments, demonstrating that IκBα increases the dissociation rate of NF-κB from the DNA in a highly efficient kinetic process. Analysis of various IκB mutant proteins shows that this process requires the C-terminal PEST sequence and the weakly folded fifth and sixth ankyrin repeats of IκBα. Mutational stabilization of these repeats reduces the efficiency with which IκBα enhances the dissociation rate.

The NF-κB transcription factors play key roles in normal growth and development, in inflammatory and immune responses, and in numerous human diseases (1, 2). The most abundant NF-κB is the p50/p65 heterodimer, but other homo- and heterodimers of p65 (RelA), RelB, c-Rel, p50, and p52 subunits are present also (1). Specific inhibitors of NF-κB transcription, including IκBα, IκBβ, and IκBε, block the transcriptional activity of p65- and c-Rel-containing NF-κB dimers (3). In resting cells, NF-κB transcriptional activity is strongly inhibited by IκBα that keep the NF-κB in the cytoplasm, preventing its nuclear localization and association with DNA (4, 5). Stress signals induce activation of IκB kinase, which phosphorylates the N-terminal signal response domain of NF-κB–bound IκBα, leading to subsequent ubiquitination and degradation by the proteasome (6). IκBα then enters the nucleus, binds DNA, and regulates transcription of its numerous target genes (7).

DNA-bound NF-κB has been detected at hundreds of genes (8) with a loosely defined consensus sequence, based on NF-κB–responsive genes, called a “κB site” (1). Thousands of such sites are present in the DNA (9, 10). NF-κB use the Rel homology domain to recognize κB sites in the DNA, but only p65, c-Rel, and RelB have transcription activation domains (11). The large number of genes that are activated by NF-κB show widely varying levels and kinetics of transcription activation and postinduction repression, but the mechanism of this diversity still is not understood (12). DNA binding specificity may not be able to explain the wide variations in transcription responses. Although NF-κB family members can form homo- and heterodimers, and purified dimers are able selectively to bind various oligonucleotides corresponding to diverse κB sites (13), crystal structures of several NF-κB homo- and heterodimers with various κB sites show few specific base contacts (14–17). Early experiments measuring DNA binding were done under nonphysiological conditions in which the binding affinity of the NF-κB for DNA was shown to be extremely high, 10−10 M−1 (18). A wide range of binding affinities, from 10−6 to 10−3 M−1, for the same κB site binding to the same NF-κB (p50/p65) heterodimer have been reported in the literature (19). Recent intracellular photobleaching experiments suggest that NF-κB dissociates from the DNA at a surprisingly rapid rate (20).

We previously showed that IκBα binds tightly to NF-κB with a Kd of 40 PM at 37°C as a consequence of a slow dissociation rate constant on the order of 10−4 s−1, which translates into a half-life of 2 h (5). The measured intracellular half-life also is on the order of several hours (21). Previously reported binding affinities in the nanomolar range for IκBα binding to NF-κB (p50/p65), obtained from gel shift and competition assays, are inconsistent with the extremely long intracellular half-life of the complex (19) as well as with the direct binding experiments (5).

Crystal structures of DNA-bound NF-κB(p50/p65) and IκBα-bound NF-κB(p50/p65) show overlapping but nonidentical binding surfaces (13, 22–24). DNA contacts the loops protruding from the dimerization and N-terminal domains of the Rel homology domain and the linker between them, whereas IκBα mainly contacts the dimerization domain and helix3-NLS-helix4 at the C terminus of the Rel homology domain of p65 (Fig. 1). The ankyrin repeat domain (ARD) of IκBα forms the main interaction surface with the dimerization domains of NF-κB (23, 24).

The IκBα gene is strongly activated by NF-κB, resulting in new synthesis of IκBα (25–27). The newly synthesized IκBα enters the nucleus and is responsible for rapid postinduction repression of NF-κB transcriptional activity (28, 29). Early experiments with IκBα showed that it was very efficient in competing with DNA for binding NF-κB (30). These results led to the suggestion that IκBα can enter the nucleus and remove NF-κB from the DNA by an “active dissociation” mechanism. However, the term “active” implies that IκBα increases the rate of dissociation of NF-κB from the DNA, but the equilibrium competition experiments cannot prove this effect (4). Rapid replacement of DNA with IκBα would be expected, based on the already rapid binding kinetics of NF-κB with DNA and the tighter binding affinity of IκBα with NF-κB, without any need to invoke an active dissociation mechanism (5).

Here, we have undertaken biophysical experiments designed to measure the association and dissociation kinetics of NF-κB binding to single κB sites in the DNA under physiological conditions. The dissociation rates recapitulate the in vivo photobleaching kinetic results (20). Surprisingly, 2 different kinetic experiments also show that IκBα increases the dissociation rate of NF-κB(p50/p65) from the DNA in a highly efficient and concentration-dependent manner. Thus, the previously proposed active dissociation mechanism, which was based on insufficient experimental evidence, does, in fact, occur. Experiments using mutants of NF-κB and IκBα suggest a mechanism in which the weakly folded ankyrin repeats (ARs) 5 and 6 of the IκBα ARD are required for this phenomenon.


The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0908797106/DCSupplemental.
Results

DNA Binding to NF-κB Is Rapid and Reversible. Previously, binding thermodynamics measurements of single κB-site DNA molecules binding to various homo- and heterodimers of NF-κB family members have shown a broad range of binding affinities from picomolar (18) to nanomolar (31) to almost micromolar (19) levels. Recently, in vivo fluorescence recovery after photobleaching experiments showed rapid kinetics of exchange of NF-κB from the DNA with a half-life of the bound NF-κB on the order of 30 s (20). We carried out real-time binding kinetics measurements using surface plasmon resonance (SPR) with purified NF-κB homo- and heterodimers of p50 and p65 and oligonucleotides containing a single κB site at physiological salt concentrations and 37 °C. We chose 6 different DNA sequences based on previous reports that different κB sites show very different kinetics of transcription activation and postinduction repression (12). Except for the urokinase promoter sequence, the NF-κB(p50/p65) heterodimers bound with observed $K_d$s in the nanomolar range. Lower affinities were observed for the NF-κB(p65/p65) homodimers, and these homodimers also showed more variable binding affinities (supporting information (SI) Table S1). The nanomolar binding affinities to specific κB DNA sites are the result of rapid association ($k_a = 1 \times 10^6 \text{M}^{-1}\text{s}^{-1}$) and dissociation ($k_d = 1.7 \times 10^{-5} \text{s}^{-1}$) rates at 37 °C under physiological conditions. To validate the SPR measurements, equilibrium and stopped-flow fluorescence was used to determine the binding association and dissociation kinetics for NF-κB(p50/p65) heterodimers binding to the IgκB site contained in a hairpin with a pyrene label at the 5′ end. Equilibrium binding experiments in which the change in fluorescence intensity was measured, carried out at 25 °C, gave a $K_d$ of 10 nM (Fig. S1). The stopped-flow kinetics experiments gave a $k_a$ of $1.2 \times 10^6 \text{M}^{-1}\text{s}^{-1}$ and a $k_d$ of 0.4 s$^{-1}$, resulting in an observed $K_d$ of 3 nM. It is relatively common for both $k_a$ and $k_d$ values to be higher when determined by fluorescence kinetics than when determined by SPR, but for overall $K_d$ values to be similar (32). A random sequence of DNA showed no binding by SPR and a dissociation rate of re-binding of the NF-κB to the DNA ($k_d$) was measured. A schematic of the binding events is shown below the graph (Fig. S1). The dissociation rate constant determined from 4 independent experiments, performed as described in (A), as a function of $I_{NaCl}$ concentration. The slope of the line (10 $M^{-1}\text{s}^{-1}$), which is the pseudo-second-order rate constant for active dissociation, indicates that NF-κB enhanced dissociation is highly efficient. A control experiment, performed as described in (A), in which varying concentrations of DNA (red, 100 nM; blue, 1000 nM) were used instead of NF-κB in the dissociation step. Even at 1000 nM DNA, only a slight difference in the dissociation rate was observed. (D) Stopped-flow fluorescence experiment in which pyrene-labeled hairpin DNA (0.25 μM) complexed to NF-κB(p50/19–363/p65/1–325) (0.5 μM) in syringe 1 was mixed rapidly with a 50-fold excess (relative to NF-κB) of either unlabeled hairpin DNA (black, $k_d = 0.41 \text{ s}^{-1}$) or IκB (red, $k_d = 18.2 \text{ s}^{-1}$).

IκB Increases the Rate of Dissociation of NF-κB from Promoter Sites. A flowing, real-time kinetics experiment using SPR was designed to probe whether IκB increases the rate of dissociation of NF-κB from the DNA. In this experiment, κB site-containing oligonucleotides were captured via a biotin–streptavidin linkage onto the sensor chip. NF-κB(p50(19–363)/p65(1–325)) was allowed to associate with the DNA until a pseudoequilibrium was reached ($t = 100 \text{ s}$). Varying concentrations of IκB were then injected through the second sample loop (co-inject experiment), and the dissociation rate constant ($k_d$) was measured. A schematic of the binding events is shown below the graph. (B) Plot of the dissociation rate constant determined from 4 independent experiments, performed as described in (A), as a function of $I_{NaCl}$ concentration. The slope of the line (10 $M^{-1}\text{s}^{-1}$), which is the pseudo-second-order rate constant for active dissociation, indicates that IκB enhanced dissociation is highly efficient. (C) A control experiment, performed as described in (A), in which varying concentrations of DNA (red, 100 nM; blue, 1000 nM) were used instead of NF-κB in the dissociation step. Even at 1000 nM DNA, only a slight difference in the dissociation rate was observed. NF-κB(p50(19–363)/p65(1–325)) was allowed to associate with the DNA until a pseudoequilibrium was reached ($t = 100 \text{ s}$). Varying concentrations of IκB were then injected through the second sample loop (co-inject experiment), and the dissociation rate constant ($k_d$) was measured. A schematic of the binding events is shown below the graph. (B) Plot of the dissociation rate constant determined from 4 independent experiments, performed as described in (A), as a function of $I_{NaCl}$ concentration. The slope of the line (10 $M^{-1}\text{s}^{-1}$), which is the pseudo-second-order rate constant for active dissociation, indicates that IκB enhanced dissociation is highly efficient. (C) A control experiment, performed as described in (A), in which varying concentrations of DNA (red, 100 nM; blue, 1000 nM) were used instead of NF-κB in the dissociation step. Even at 1000 nM DNA, only a slight difference in the dissociation rate was observed. (D) Stopped-flow fluorescence experiment in which pyrene-labeled hairpin DNA (0.25 μM) complexed to NF-κB(p50(19–363)/p65(1–325) (0.5 μM) in syringe 1 was mixed rapidly with a 50-fold excess (relative to NF-κB) of either unlabeled hairpin DNA (black, $k_d = 0.41 \text{ s}^{-1}$) or IκB (red, $k_d = 18.2 \text{ s}^{-1}$).
addition, when NF-κB was immobilized and IκBα was bound, no effect on the IκBα dissociation rate was observed even at high concentrations of κB-site DNA. Stopped-flow fluorescence experiments also were performed in which the NF-κB-pyrene hairpin DNA complex was dissociated with a 50-fold molar excess of either unlabelled DNA or IκBα. Again, IκBα caused a dramatic 45-fold increase in the dissociation rate from 0.4 s⁻¹ to 18 s⁻¹, whereas the unlabelled DNA had no effect (Fig. 2D).

**Residues 305–325 of NF-κB (Helix 4 of the NLS Extension) Are Required for IκBα-Mediated Dissociation.** Truncation mutants were constructed to ascertain which parts of the NF-κB and IκBα molecules were important for IκBα-mediated dissociation. Initially, the NF-κB (p65) was truncated at residue 304, thus deleting helix 4 just after the NLS sequence. Although this protein formed heterodimers and bound to κB-site DNA with the same affinity as wild type, IκBα did not enhance dissociation if residues 305–325 were missing from p65 of the NF-κB heterodimer (Fig. 3A). This result was not satisfying, however, because deletion of residues 305–325 causes a dramatic loss of binding affinity of NF-κB for IκBα (5). To probe more subtly the role of the NF-κB(p65) NLS interaction with IκBα in facilitating dissociation, Arg 304 in the p65 NLS was mutated to Ala. This mutation reduced IκBα-NF-κB binding affinity by 2.2-fold and reduced the IκBα-mediated dissociation by 1.5-fold (Fig. 3B).

We previously had shown that truncation of residues 288–317 of IκBα had no effect on NF-κB binding nor did phosphorylation of the PEST sequence (5, 34). As expected, IκBα (67–317) and PEST-phosphorylated IκBα (67–317) also efficiently mediated dissociation. Deletion of residues 282–287 to truncate partially the IκBα PEST sequence significantly reduced the efficiency of IκBα-mediated dissociation of NF-κB from the DNA. Deletion of residues 275–287 to remove the PEST sequence completely had an even larger effect (Table S2A and Fig. 3C). These results strongly implicate the negatively charged PEST sequence in removal of NF-κB from the DNA.

The Weakly-Folded Fifth and Sixth Repeats Are Critical for IκBα-Mediated Dissociation. IκBα does not conform to the consensus for stable ARDs, and we have previously shown that mutation back to the consensus sequence for stable ARDs stabilizes IκBα (35, 36). All the stabilized mutants were less able to increase the dissociation of NF-κB(p50/p65) from the DNA despite widely varying NF-κB binding affinities (Table S2B and Fig. 4). Mutation of Y254L/Q255H weakened the binding by 100-fold, but this protein still was effective in increasing the dissociation of NF-κB from the DNA (Table S2B). In contrast, mutation of Y254L/T257A, which prefolds the sixth repeat, weakened binding only 30-fold but was much less efficient in increasing dissociation (36). The C186P/A220P mutant bound to NF-κB with the same binding kinetics and affinity (Table S2B) but was less efficient at increasing dissociation of NF-κB. The C186P/A220P mutant was less able to enhance dissociation on 3 different κB promoter sequences, indicating that enhanced dissociation is DNA sequence-independent (Fig. 4C).

**Discussion**

**NF-κB-DNA Binding Constants.** As cited in the Introduction, several widely varying values have been reported for the affinity of the interaction between NF-κB and DNA and between NF-κB and IκBα. Although it is not possible to replicate physiological conditions exactly, it now has become clear that the binding affinity of NF-κB for a single canonical κB site is 3–10 nM. Protein–DNA interactions are very sensitive to ionic strength (37), and the anomalously low Kᵦ of 0.1 nM probably reflects the low salt concentration used in these experiments (18). We have no explanation for the much weaker binding affinities measured by fluorescence anisotropy (19). The nanomolar binding affinity for κB-site DNA is derived from fast-on, fast-off kinetics, and these results are fully consistent with intracellular measurements (20). Given that the dissociation of NF-κB from the DNA already is relatively rapid, it was surprising that IκBα significantly enhances the dissociation rate even further. Why and how IκBα markedly increases the NF-κB-DNA dissociation rate is discussed further in the following sections.
NF-κB Has Overlapping but Nonidentical Binding Sites for DNA and IκBα. Although several different mechanisms of transcriptional inhibition have been proposed, including the formation of an inhibited ternary complex, IκBα does not work by such a mechanism. Crystal structures of NF-κB(p50/p65) bound with either DNA or IκBα show that the binding sites on NF-κB for DNA and for IκBα are overlapping but are not identical, and binding of DNA or IκBα to NF-κB is known to be mutually exclusive. IκBα mainly contacts the NLS and the dimerization domains, whereas DNA mainly contacts the interface between the dimerization domains and the N-terminal domains (Fig. 1). The very high binding affinity appears to be almost entirely the result of small regions of the binding interface at either end of the elongated contact surface, resulting in a large folding enthalpy contribution to the binding affinity (5, 34). Truncation of the NLS domain of NF-κB(p65) (residues 291–325) results in a 5,000-fold decrease in binding, and this polypeptide alone binds to NF-κB with an affinity of 1 μM (34). At the other end of the interface, ARs 5 and 6 are not fully folded in free IκBα, but they fold upon binding to NF-κB (38). In addition, the PEST region, which also is critical for binding, is negatively charged, like the DNA, and might enhance DNA dissociation from NF-κB partly via electrostatic repulsion.

Structural Model of IκBα-Mediated Active Dissociation. A possible model of the enhanced dissociation process was developed based on the mutational studies (Fig. 5). In this model, when IκBα approaches the DNA-bound NF-κB, it interacts first with the p65 NLS, which does not participate in binding to DNA and is thus available for ternary complex formation (14–16, 22). Deletion of residues 305–321 of NF-κB(p65) did not affect NF-κB-DNA binding but abolished the ability of IκBα to enhance NF-κB dissociation from the DNA. Theoretical studies predicted that mutation of Arg-304 to Ala in the NLS would reduce IκBα-NF-κB binding affinity (39), and indeed, this mutation reduced affinity by approximately the same amount that it reduced the active dissociation. These observations support the hypothesis that interaction with the NLS is the first step (Fig. 5). With its 1-μM binding affinity, the interaction of the first and second ARs of IκBα with this “NLS polypeptide” region of NF-κB(p65) would be sufficient to form a small amount of short-lived ternary complex. The formation of ternary complexes between other IκB proteins and their respective NF-κBs and DNA has been predicted and observed (40, 41). Subsequently, IκBα may dissociate from the ternary complex, or it may interact further with NF-κB causing its dissociation from the DNA.

The weakly folded C-terminal part of the IκBα ARD is critical to its ability to promote dissociation of NF-κB from the DNA. This region folds onto the dimerization domains of the NF-κB (p50/p65) and shares a binding interface with the DNA. We previously showed that ARs 5 and 6 of IκBα are weakly folded (38, 42, 43) and that mutations that restore the consensus for stable ARs can promote folding of this region (35, 36). The stabilizing mutations had varied effects on NF-κB-IκBα binding, but all showed reduced ability to promote dissociation of NF-κB from the DNA. The C186P/A220P mutation bound to NF-κB with the same affinity but was significantly less able to enhance NF-κB dissociation from the DNA.

Kinetic Model of IκBα-Mediated Active Dissociation. To explore whether kinetic enhancement would be important under physiological conditions, we built an ordinary differential equation model using some parameters derived from a validated computational model of NF-κB signaling (29). Quantitative models of NF-κB signaling have been built, but these models do not explicitly consider the κB-site-NFκB interaction because the kinetic rate constants remain uncertain (29). We assumed that there is an excess of κB DNA binding sites in the genome such that all nuclear NF-κB can bind to DNA, as is observed experimentally. This situation is, in fact, mimicked by the experimental conditions of the SPR experiments described in earlier sections. The model contains the 3 species: NF-κB, DNA, and IκBα. NF-κB can bind to the DNA, unbind, and form a complex with IκBα. Additionally, IκBα can bind to the NF-κB-DNA complex forming a transient ternary complex (estimated KD ≈ 1 μM), but this model gave results equivalent to the simpler model shown in Fig. 6A. The active removal of NF-κB from the DNA complex by IκBα is determined by the parameter, ka.

The rate constants governing the NF-κB-DNA complex (ka, k0) were determined by SPR (5) and were validated by intracellular experiments (21). Simulation of the SPR experiment itself, following the addition of 100 nM of NFκB to the DNA, resulted in rapid formation of an NFκB-DNA complex. At time 0, one can calculate the concentration [IκBα] of IκBα as the system akin to the flowing IκBα into the flowcell of the Biacore instrument. The model simulations recapitulate the experimental observation that IκBα accelerates the dissociation of the NF-κB-IκBα-DNA complex in a concentration-dependent manner (Fig. 6B). The half-life of the NF-κB-DNA complex was derived from simulations carried out using physiological concentrations of DNA and proteins, and a color scale plot was generated to reveal the dissociation characteristics of the NFκB-DNA complex as a function of the IκBα-dependant and IκBα-independent dissociation rate constants (Fig. 6C). The model simulations show that active dissociation by IκBα has significant impact on the effective NFκB-DNA complex half-life for a wide range of NFκB-DNA dissociation rates, even when the IκBα-independent off rate is as much as 16 times faster than the experimentally measured k0. The apparent robustness underscores the likely physiological importance of IκBα-enhanced dissociation of NFκB from κB sites.

Kinetic enhancement of dissociation is expected to increase dramatically the effectiveness of newly synthesized IκBα in causing transcriptional repression before an equilibrium concentration is reached that is sufficiently high to compete effectively for κB sites in the DNA. Thus, new synthesis of even
a small amount of IxBα would be expected to reduce NF-κB transcriptional activity significantly. Enhanced dissociation by IxBα may play an even more critical role in turning off transcription from genes that have multiple κB sites (45) or when transcription co-activators increase the affinity of transcription factors for some sites in the DNA (46). To understand the extent to which active dissociation operates under physiological conditions, measurements of IxBα-mediated active dissociation of NF-κB from specific promoters in cells will be required.

**Experimental Procedures**

**Protein Expression and Purification.** Human wild-type IxBα (28–287) and mutant and truncated forms induced by QuikChange (Stratagene) mutagenesis were expressed at 20 °C in Escherichia coli BL21 DE3 cells and purified using a Hi-load Q Sepharose (GE Healthcare) followed by a Superdex 75 column (GE Healthcare), as described previously (5, 43). The protein concentrations were determined by spectrophotometry, using a molar absorptivity of 12,090 M–1 cm–1.

NF-κB proteins were expressed in E. coli (BL21) DE3 cells at room temperature and were purified by a tandem Q then S Sepharose column (GE Healthcare) and finally by size exclusion on an S-200 column (GE Healthcare). Proteins purified by the 2 different methods were confirmed by mass spectrometry.

**SPR Experiments.** Sensorgrams were recorded on a Biacore 3000 (GE Healthcare) using streptavidin chips. For IxBα binding experiments, biotinylated NF-κB (p65) was prepared as already described (5). Homo- and heterodimers were formed by incubating the biotinylated NF-κB (p65) with a large excess of the other, unbiotinylated subunit in vitro for 1 h at 25 °C and overnight at 4 °C and were captured on the surface of the streptavidin sensor chip as previously described (5). IxBα-binding data were collected in 150 mM NaCl, 10 mM MOPS (pH 7.5), 10% (v/v) glycerol, 3 mM dithiothreitol, 0.5 mM sodium azide, 0.2 mM EDTA, and 0.005% P20 (GE Healthcare) were captured on the surface of the streptavidin sensor chip using manual inject.

**Fluorescence Studies.** All fluorescence experiments used a pyrene-labeled hairpin DNA: 5′-AmMC6/GGGAAATTCTCCCCCGAATTCCTCC-3′ (IDT Technologies) corresponding to the IκB-κB site (GGGAAATTC). The hairpin DNA (20 nmol in 75 µL of 0.1 M sodium tetraborate (borax), pH 8.5) was labeled at the 5′ end (p65) with 1-pyrenenbutyric acid N-hydroxysuccinimide ester (14 µL of a 9-mg/mL solution in DMSO; Sigma-Aldrich) at the AmMCl group at 25 °C for 6 h. The reaction was quenched by addition of 1 mL ethanol, and the labeled DNA was purified by C18 RP-HPLC in 20 mM ammonium acetate, pH 6.5, with a 60-min gradient from 0 to 60% acetonitrile. The oligonucleotide and the protein were dissolved in 25 mM Tris, 150 mM NaCl, 0.5 mM EDTA, and 1 mM DTT at pH 7.5 and 25 °C.

**DNA NF-κB binding constant titration measurements were performed using a photon-counting fluorimeter (FluoroMax-P). Samples were incubated at 25 °C for 3 min before the start of each experiment. Kd determination was performed with a constant final concentration of the pyrene-DNA in a 1-µL cuvette (5 nM in 1-mL final volume) to which various concentrations of NF-κB were added. The sample was exited at 346 nm (2-nm slit), and the emission was monitored at 377 nm (5-nm slit) with an integration time of 2 s and a 3-min equilibration time. For each NF-κB concentration, the fluorescence intensity of a blank sample in Tris buffer was subtracted from the labeled DNA-containing sample. The data were fitted (Kaleidagraph software 4.0, Synergy, Inc.) to Y = m1*(1 + m2 + m3) + m2*exp(-m3*X)/m3 where Y corresponds to the maximum fluorescence, m1 is the amplitude, m2 is the Kd, m3 is the pyrene-DNA concentration, and X is the NF-κB concentration.

Rapid kinetics experiments were performed at 25 °C on a Biologic SFM-20 stopped-flow fluorimeter. The mixing volume was 120 µL with a sampling period of 200 µs to 5 ms. The association kinetics for the pyrene-DNA were measured in triplicate at 10 concentrations of NF-κB heterodimer (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, 1.1, 1.5, and 3 µM) maintaining a constant concentration of pyrene-DNA (0.1 µM before mixing). The association rate constant was obtained from a single exponential fit (Kaleidagraph, Synergy, Inc.).

The dissociation rate constant of the NF-κB-pyrene DNA complex was measured using a stopped-flow fluorimeter (in a 2:1 ratio; final NF-κB concentration of 50 nM) by adding an excess of unlabeled pyrene DNA (1:10, 1:50, and 1:100, NF-κB-pyrene DNA). The experiment was repeated using IxBα(28–287) instead of unlabeled DNA hairpin in the same concentrations and ratios. For both experiments, the data were fit (Kaleidagraph) to the equation m1 + m2*exp(-m3*X) where m1 = end point, m2 = amplitude, and m3 = Kd.

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Paper 2

Oscillations are commonly observed in cellular behavior and span a wide range of timescales, from seconds in calcium signaling to 24 hours in circadian rhythms. In between lie oscillations with time periods of 1–5 hours seen in NF-κB, p53 and Wnt signaling, which play key roles in the immune system, cell growth/death and embryo development, respectively. In the first part of this article, we provide a brief overview of simple deterministic models of oscillations. In particular, we explain the mechanism of saturated degradation that has been used to model oscillations in the NF-κB, p53 and Wnt systems. The second part deals with the potential physiological role of oscillations. We use the simple models described earlier to explore whether oscillatory signals can encode more information than steady-state signals. We then discuss a few simple genetic circuits that could decode information stored in the average, amplitude or frequency of oscillations. The presence of frequency-detector circuit downstream of NF-κB or p53 would be a strong clue that oscillations are important for the physiological response of these signaling systems.

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Minimum ingredients for generating oscillations

The most obvious examples of periodic behavior are circadian rhythms [1,2] and cell cycles [3]. Much more rapid oscillations are seen in the levels of cellular calcium [4]. Many hormones also show intermittently periodic behavior and pulsatile secretion [5]. Figure 1 shows three systems where oscillations have been observed: the NF-κB [6**,7**,8,9], p53 [10**,11**] and somitogenesis systems [12,13**,14], which are important for the immune response, cell growth/death and embryo development, respectively.

The purpose of this review is to provide a brief conceptual overview of deterministic mathematical models of such oscillations, and suggest how they can be used to explore the potential physiological role of oscillatory signals. Our emphasis will be on simplified models and the quintessential understanding they provide [15–17], rather than complex models which aim to reproduce experimental data in detail.

Modeling oscillations

Introduction

Living cells continuously adjust gene expression patterns in response to changing environmental conditions. A simple way of encoding the presence of a stress or stimulus is to shift the concentration of a signaling molecule from one steady-state level to another. This scheme has potential disadvantages including the cost of continuous production of signaling molecules at a high level and unwanted cross-talk between pathways. It is not surprising, therefore, that cells often encode information about environmental changes in complex time-varying signals. This review deals with one subclass of such systems: those that exhibit oscillations.
Figure 1

The NF-κB, p53 and Wnt signaling systems. Schematic figures of some of the important proteins (rounded rectangles), mRNA (rectangles), genes (double lines), and interactions (activatory: ordinary arrows; inhibitory: barred arrows) in the three systems. Ordinary arrows are also used to represent conversion and transport between cellular compartments, and merging arrows indicate complex formation. More details of each network can be found in Refs. [59,60] (NF-κB), [61,62,53] (p53), [63,64] (Wnt). Experiments showing sustained and damped oscillations in these systems can be found in Refs. [6,C15/C15,7,C15/C15,8,9] (NFκB), [10,C15/C15,11,C15/C15,13,C15/C15,14] (p53), [13,C15/C15,14] (Wnt).
1. Processes that take a minimum amount of time. For example, transcription and translation (Figure 2b.1).
2. Many intermediate steps, that is, a long feedback loop. Each step adds to the overall time delay (Figure 2b.2).
3. Switch-like responses, where a regulator must reach a threshold concentration before it acts on the next in the loop (Figure 2b.3).
4. Saturated degradation, where the degradation of a regulator is delayed by saturated complex formation (Figure 2b.4).
Modeling negative feedback and time delay

The model of Hes oscillations in [19] is an example of case (1) above, where an explicit time delay in the production of Hes mRNA (which is inhibited by Hes protein) represents the amount of time taken for transcription, translation and then nuclear import of Hes protein (the identical model was published again in [20]). Models using explicit time delay have been widely used to model oscillations, for example, in respiration patterns and hematopoiesis [21], p53 [22], somitogenesis [23], insulin secretion [24], and the hypothalamic-pituitary-adrenal axis [25]. In our opinion, however, using explicit delays is somewhat ad hoc and we find it more satisfying to model the specific molecular processes that produce delay.

The conceptually simplest way to do this is case (2) above. For example, an oscillator formed by a loop of six nodes, involving three proteins, and their mRNA, each protein inhibiting transcription of the next (Figure 2b.2), has been modeled and experimentally realized in Ref. [26]. Another synthetic circuit that oscillates due to many intermediate steps was constructed in [27]. More complex is case (3) where one regulator affects only the next when it reaches a threshold concentration. The delay arises from the time taken for the threshold to be reached. Simple models of oscillations have implemented this using either a highly cooperative interaction with a large (≥8) Hill coefficient [28] or positive feedback loops [29,30]. Both large Hill coefficients and positive feedback result in sigmoidal, switch-like responses and hence a threshold concentration below which the response is essentially zero. Combining positive and negative feedback has the advantage of making oscillations more robust and yet tunable [29,30], and has been used to model many phenomena, including cell cycles [31,32], circadian rhythms [2,16,33], division site localization in *Escherichia coli* [34], and p53 oscillations [10,35,36], as well as to design synthetic oscillators [37,38].

The next section elaborates on case (4), saturated degradation, which we find particularly interesting as it is seen in NF-κB, p53 and Wnt signaling. Note however that these time-delay mechanisms are not mutually exclusive. Systems typically use several of these mechanisms, each contributing to the overall delay.

**Saturated degradation models of NF-κB, p53 and Wnt signaling**

The key negative feedback loop underlying sustained and damped oscillations in NF-κB, in both wild-type and genetically modified cells, involves the inhibitor protein IκBα. The feedback loop has two legs (Figure 2c): first, NF-κB activates IκBα production, second, IκBα inhibits NF-κB by binding to it and sequestering it in the cytoplasm. Leg (i) is active when there is little IκBα, so most NF-κB is free to enter the nucleus, causing IκBα levels to rise. Free NF-κB levels then fall rapidly as it gets bound to newly synthesized IκBα. In the model of Figure 2c [39], the amount of NF-κB–IκBα complex has a Michaelis-Menten form: \( N_c I/(K_I + I) \), where I is the IκBα concentration and \( N_c \) is the total cytoplasmic NF-κB concentration. The binding is strong, that is, \( K_I \) is small, so IκBα levels quickly become large enough to saturate NF-κB, at which point the amount of complex becomes equal to \( N_c \) and independent of I. This is leg (ii) of the feedback. Now there is no further production of IκBα, so its levels will eventually fall. However, IκBα molecules that are bound to NF-κB are more susceptible to IKK-dependent degradation (due to stabilization of IκBα by NF-κB [40]) so the degradation rate (second term in the d[dt] equation in Figure 2c) depends not on the amount of IκBα present, but on the amount of the complex. Because the complex is saturated and equal to \( N_c \) most of the time (see blue curve in Figure 2c, bottom panel) we call this ‘saturated degradation’. If, instead, IKK-inducing stimuli led to equal degradation of both free and complexed IκBα, then the degradation rate would be proportional to I, which would make I fall exponentially fast. By contrast, with saturated degradation I falls slower than exponentially, resulting in the more rounded shape of I vs. time seen in Figure 2c (green curve). This provides a sufficient time delay to generate oscillations. The model of Figure 2c is of course a simplified one and it is important to check whether the assumptions made in simplifying the system are reasonable. For example, in wild-type cells, free IκBα is also degraded but the model ignores this. This could be included in the model as an additional degradation term, that is, proportional to I leading to an exponential, non-saturated, decrease of IκBα levels, which may neutralize the time delay provided by the saturated degradation pathway. To see which pathway of degradation is more important, one must compare the half-life of free IκBα with the rate of the NF-κB–IκBα complex formation. Using numbers for wild-type cells from Ref. [6,41], we find the average time for complex formation is of the order of tens of seconds, whereas the half-life of free IκBα is several minutes. Thus, we expect that saturated degradation is an important source of time delay in the NF-κB–IκBα feedback loop despite the presence of other non-saturated degradation pathways.

The p53-Mdm2 feedback loop (Figure 2d) is very similar: first, p53 activates Mdm2 production, second, Mdm2 inhibits p53 by binding to it. Mdm2 also causes poly-ubiquitination and thereby degradation of p53, again resulting in saturated degradation. Here, it is the transcription factor (TF) that has saturated degradation, rather than the inhibitor. This is the opposite to what happens with NF-κB, but the model of [42], a simplified version of which is shown in Figure 2d, shows that it does not matter for generating oscillations — the
time delay in this model occurs when p53 levels are high and fall relatively slowly, rather than when the inhibitor levels are high (see Figure 2d). Because p53 levels remain high for longer, Mdm2 levels also rise much higher than they would without saturated degradation. The extra time required for Mdm2 levels to fall in leg (ii) of the feedback provides an additional delay that helps oscillations. The half-life of p53 in the absence of Mdm2 is of the order of hours, whereas the average time for p53-Mdm2 complex formation is of the order of seconds (see [42] and references therein). So again, even though the p53-Mdm2 interaction is quite transient, we expect the saturated complex formation to be the dominant pathway for p53 degradation, rather than non-saturated pathways.

A third system where saturated degradation has been used to model oscillations is Wnt signaling (Figure 2e) [43,44]. Wnt and β-catenin are upstream controllers of all oscillating signals in the presomitic mesoderm [45] so it is useful to study the negative feedback loop involving Wnt, β-catenin and Axin2. The model of this loop in Figure 2e demonstrates an interesting variation on the ones in Figure 2c,d, showing that saturated degradation need not arise from the same complex that results in inhibition of the TF. In the model, Axin2 binds to the TF β-catenin and separately to the Wnt-activated LRP receptor complex. The former provides the negative feedback but the binding is weak and not saturated. The latter complex has a much smaller dissociation constant, that is, larger binding strength, and results in saturated degradation of Axin2. Thus, negative feedback and saturated degradation are separate ingredients that can be implemented independently in an oscillator.

Other simple models of oscillations using saturated degradation can be found in Refs. [46–49]. It is interesting that saturated degradation typically produces quite spiky oscillations, which have the advantage that the signaling molecule can achieve high levels without having to be produced at a high rate all the time. This brings us to the question of what these oscillations are useful for.

Potential physiological role of oscillations
In some systems periodicity is an obvious requirement. A periodic spatial pattern is clearly necessary for proper somite spacing and temporal oscillations in Wnt and Notch targets are a way of generating the spatial periodicity [50–51]. Circadian clocks in cyanobacteria are useful for entraining metabolism, photosynthesis, cell division and global gene expression to the day–night cycle [52]. However, in NF-κB and p53 it is not obvious that the oscillations per se are important for the physiological response. For example, it has been suggested that p53 pulses might be a byproduct of pulsatility in ATM, an upstream regulator of p53 required for proper DNA damage repair [53].

From the opposite angle, what benefit could oscillations provide in helping NF-κB and p53 produce gene expression patterns specific to distinct stimuli? One idea is that signals with complex temporal variation contain more information than steady-state signals and therefore can control downstream genes more subtly [39,54,53]. We elaborate on this below.

Encoding information in oscillatory signals
Steady-state signals have a single adjustable characteristic, the level of the signal, while oscillations have many — average, amplitude, time period, spikiness, spike width, spike symmetry (see Figure 3a). Oscillations in NF-κB or p53 could thus encode more information than steady-states about which stimulus was triggering the system provided: first, different stimuli affect different parameters, and second, changing different parameters affects oscillation characteristics differently.

(i) In the p53 system, we know that different stimuli affect different sets of parameters. DNA damage affects Mdm2 activity and stability, hypoxia additionally alters the transcription rate of Mdm2, while other triggers like Nutlin change only the binding strength between p53 and Mdm2 (see references in [42]). In the NF-κB model, many triggers act through the IKK level which affects the degradation rate of 1κBα, but different stimuli produce different profiles of IKK and thereby NF-κB [55,56].

(ii) Figure 3b–e show that for the simple model of NF-κB described above, changing different parameters does indeed affect oscillation characteristics differently. The plots show that there are parameter regimes where one of the three characteristics, time period, average and peak, can vary while the other two remain constant. However, not all characteristics can be independently varied because there are correlations. For example, spikiness is correlated with larger time periods, lower averages and asymmetry of the spike shape. Experiments have also shown that characteristics of the temporal profile of nuclear NF-κB concentration, for example, the steepness of the initial increase and the later decline, are under the control of different regulators [56*].

Similar behavior is seen in the p53 and Wnt models, so one can conclude that an oscillatory signal produced from a simple negative feedback loop with saturated degradation can indeed encode more information than steady-state signals.

Decoding information from oscillatory signals
Next, it is necessary that different genes should respond to different characteristics of the oscillations. Consider
the simplest case where an oscillating TF binds to a single operator site (Figure 4a). Two parameters describe the binding: the association ($k_{on}$) and dissociation ($k_{off}$) rate constants. Figure 4a shows that the expression of a gene, $G_{fast}$, with sufficiently large $k_{on}$ and $k_{off}$, will closely follow the oscillations. If the stimulus changes the peak level of the oscillations, the expression of this gene will follow that change; $G_{fast}$ is a peak-detector. By contrast, the activity of $G_{slow}$, which has a much smaller $k_{off}$, will not follow the oscillations because its expression has not time to decline much before the next spike occurs. Thus, this gene’s expression averages over many spikes. $G_{slow}$ is therefore an average detector. Note that if we look at the concentration of the proteins encoded by $G_{fast}$ and $G_{slow}$, then we also have to take into account their half-lives and those of the mRNAs. Thus, for example, if $G_{fast}$ produces a very long lived mRNA or protein then the protein concentration would follow changes in the average of the input oscillations rather than the peak. By contrast, even if $G_{slow}$ produces a short-lived mRNA or protein it would remain an average detector.

The slightly more complex circuit of Figure 4b is a frequency detector. Two genes with twofold different $k_{off}$ values respectively activate and inhibit a third, output gene. The average steady-state expression of this output gene has a maximum for a certain ‘resonance’ frequency (Figure 4b). Away from this resonance, especially for larger frequencies, the steady-state output falls dramatically. Here, the protein level will also show a similar resonance irrespective of its half-life because, assuming there is no complex translational or post-translational regulation, the average steady-state concentration of a protein is proportional to the average steady-state expression level of its gene. Interestingly, the spiky nature of the input oscillations is very important for this frequency resonance. Smooth oscillations with exactly the same time period and amplitude show a much weaker
steady-state response. This circuit is therefore a spikiness-detector as well.

**Outlook**

Some of the interesting questions this discussion raises for future research on oscillatory control are:

(A) Relating to encoding information in oscillations:

- Which parameters, and which characteristics of oscillations, do different stimuli affect in the NF-κB and p53 systems?

- Can additional feedback loops enhance the encoding abilities of oscillations?

(B) Relating to decoding information from oscillations:

- Can other decoding circuits be constructed to count, say, the number of spikes in a signal, or distinguish between symmetry and asymmetry, or other characteristics of oscillations?

- Do any such circuits exist downstream of NF-κB or p53?

The ideal experiment to evaluate the necessity of oscillations would require being able to control the frequency, number and width of spikes produced when NF-κB or p53 is triggered, and to see how this affects the physiological response. Exactly such experiments have shown that varying the frequency of oscillations in calcium signaling [4] and hormone secretion [5] changes the physiological response. Similar experiments on the NF-κB system are just beginning to be actualized, and have reported some dependence of gene expression on frequency of oscillations [57**]. Other experiments have shown the opposite, that expression of some NF-κB targets is unaffected in non-oscillating mutants [58]. These results are not necessarily contradictory, but until more comprehensive experiments become feasible for the NF-κB and p53 systems it might be useful to examine more carefully the genetic circuits downstream of these TFs. A frequency-detector circuit downstream of NF-κB or p53 would be a strong clue that oscillations are important for the physiological response.

\[ k_{on} = 0.1 \text{ min}^{-1} \text{ per operator site}, \quad k_{off} = 0.06 \text{ min}^{-1} \] respectively activate and inhibit a third, output gene G: \[ \frac{dG}{dt} = k(G_i/G_{1}(1 + G/K_{1}))(1/(1 + (G_{2}/K_{2})^{1/2})) - \gamma G. \] For this circuit, we can find parameter values for the \( \frac{dG}{dt} \) equation such that the average expression of G has a maximum (‘resonance’) when the time period is \( T = 150 \) min, when the input is spiky square-pulse oscillations (green curve). The position of this maximum can be tuned by varying \( k_{on} \) values of \( G_{1} \) and \( G_{2} \). With the same parameter values, when the input is smooth sine-wave oscillations of the same amplitude the response is much weaker (blue curve). See [47,71] for other frequency-detector circuits, involving protein phosphorylation.
Acknowledgements
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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest


Paper 3

Nested Feedback Loops in Gene Regulation

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Abstract
Protein activities may exhibit oscillations which often occur due to the existence of a negative feedback loop with an effective time delay. Many biological systems are however not limited to one feedback loop but consist of multiple loops on different regulatory levels. Here we analyze the properties of oscillations generated by one fast feedback nested within a slower feedback. An example of such a regulatory structure is the NF-κB signaling system. The nuclear-cytoplasmic shuttling of NF-κB is characterized by single cell 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 the role of the local IκBα feedback is known to drive the oscillations, the precise role of the up-stream negative feedback loops remains to be elucidated. Using NF-κB-inspired model loops we find that the design based on nested feedback loops allows for independent control of the oscillation period. Based on these results we predict that by adjusting the expression level of A20, Cezanne or other proteins functioning up-stream of IKK, e.g. by siRNA, the oscillation period can be changed by up to a factor 2.

Background
A cell must constantly relate and respond to the surrounding environment through gene expression or other regulatory mechanisms. This regulation often relies on clusters of multiple interconnected feedback loops [1, 2, 3], which in some cases makes the response more robust. For example: clustered negative feedbacks allowing for a robust adaptation [4] or interlinked fast and slow positive feedbacks adding to the robustness of a switch[1]. In other cases the combined effect of several feedback loops is better able to regulate and...
tune a systems response [5]. In *Bacillus subtilis* two positive feedback loops provide regulation and robustness to the gene expression [6] and adaptive response otherwise mainly found in negative feedback circuits [7].

Here we investigate the case where feedback loops are arranged in such a way that they do not all function on the same level of regulation: one group acting directly on the transcription factor and one group acting up-stream. We refer to this type of topology as ‘nested’ feedback loops, see fig. 1A. This configuration is inspired by the NF-κB regulatory system. NF-κB has several feedback loops but we are here focusing on the central feedback from IκBα, driving the oscillations in nuclear NF-κB activity, situated within the feedback of the A20-like proteins acting up-stream of the IκB kinase (IKK). This will be described in more detail below.

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 [8]. Nuclear translocation of NF-κB, necessary for its transcription factor 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 [9, 10].

NF-κB is regulated by, among others, the two inhibitor proteins IκBα and IκBε which bind the transcription factor and export it out into the cytoplasm. Stimulation by 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 hundreds of genes, some of which code for the inhibitor proteins, IκBε and IκBα, and A20-like proteins. In turn, the IκB proteins inhibit the NF-κB transcription factor by actively exporting it out of the nucleus. Here we omit both the negative feedback from IκBε and other inhibitor proteins and only focus on IκBα driving the oscillations.

A20 feedback acts up-stream 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 [11]. The A20 family of deubiquitinating enzymes includes A20, CYLD, Cezanne and others. Cells deficient in A20 like proteins show persistent IKK activity and elevated late phase NF-κB activity and develop severe inflammation and cachexia [12, 13, 14, 15, 16, 17].
Modeling NF-κB nuclear response

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 [18, 19]. The first computational model of the NF-κB pathway was proposed in Hoffmann et. al [20] and used to understand the dynamical responses of the NF-κB wild-type and IkB knockouts, e.g. oscillations and their absence in knockouts. This model has later been modified and used by Nelson et al [9] to analyze oscillations in single cells. Krishna et al 2006 [21] showed that the model can be significantly reduced while still capturing the essential dynamical features, in particular showing spiky oscillations in single cells.

Achieving strong quantitative agreement with bulk data requires a detailed model as presented by Werner et al [11]. Here they have extended their previous model [20] capturing nuclear NF-κB activity in bulk by including IkB β, IkB ε and A20 feedback loops. Using this model they show how A20 lowers the end level of nuclear NF-κB activity thereby providing control of the late phase of the NF-κB response. Constitutive expression of A20 alone is sufficient for this effect.

Other studies have focused on details of where and how A20-like proteins act in the pathway [22]. To address the discrepancy between bulk and single cell data, Ref. [23, 22] introduced stochasticity and showed that averaging single cell stochastic dynamics leads to a smooth damped response in bulk. Additionally Ref. [24] showed how fluctuations in the level of IKK can produce cell to cell variation in the NF-κB period and amplitude leading to a damped bulk response. Using the proposed model we aim to investigate the role of A20-like proteins as modifiers of the NF-κB oscillatory behavior in single cells.

Model

Here we propose a simple model by extending the model presented in ref. [21] to include the up-stream feedback from A20. We focus on the nested feedbacks provided by the NF-κB induced proteins in single cells and having a simple model will allow for more direct understanding of the dynamics. Fig. 1A shows a schematic representation of the model, consisting of two negative feedback loops centered around NF-κB: IkBa and A20-like proteins. We will henceforth denote the up-stream feedback ‘A20’, but it should
be seen as a group of A20-like proteins all functioning at this level of regulation, including A20, CYLD [25] and Cezanne [26, 17]. We are making a deterministic model of a single cell treated with an incoming stimulus, TNF. IκBε has from bulk experiments been shown to dampen the oscillatory nuclear NF-κB activity driven by the fast feedback from IκBα [27]. This dampening effect has however not been seen on single cell level where IκBε is thought to de-synchronize the oscillation resulting in an overall damped nuclear NF-κB response [22]. Both in bulk and single cell experiments the late onset of IκBε transcription is seen, which was already in shown in [27] and in [22] proven to be due to the later assembly of the transcription machinery and thus the onset of the RNA polymerase which is delaying IκBε mRNA with 37 min relative to the immediate synthesis of IκBα mRNA. We are here only modeling a single cell and have chosen not to include the effect of IκBε de-synchronizing as this is only relevant for larger cell averaging models. If IκBε is included the delay and the slower protein degradation would result in a weak dampening of the nuclear NF-κB activity. Our model thus remains within the spirit of the earlier minimal model, Ref. [21].

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. [22] when modeling 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, top. Active IKK can turn itself off and go back into the neutral state before being activated again by the TNF signal. IKK is shut down by A20 which inhibits the transformation from inactive to neutral IKK thereby leaving IKK in an inactive state, see Fig. 2A.

The first 30 minutes of the IKK adaption-like temporal profile in response to TNF stimulation is fitted with parameter values such that IKK peaks after about 15 minutes and then goes to a new steady state after 30 minutes of TNF induction. This part of the profile appears to be independent of A20 regulation [11]. The new steady state is however determined by A20: it is high in the absence of A20 [11] and decreases with increasing concentrations of A20. A20 protein affects only the late phase of the IKK temporal profile: the A20 mRNA level reaches maximum at 30 min and the effect of the protein is seen only after 45-60 min [28, 11]. This feature is reproduced by the model where the level of IKK in the late phase is pushed 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. This is a slow 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 them 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 90-120 min.

2. Mutants with IκBα alone show enhanced oscillations.

3. NF-κB resting level is lowered by A20.

Results and Discussion

A20-like proteins change the period of nuclear NF-κB oscillations

The basic response of our model to a continuous stimulation of TNF is oscillations of nuclear NF-κB with a time period of about 100 minutes. The original wild type response as well as IκBα and A20 knockout matches the experimental observations (see Fig. 2 and the interactive applet [29]). Thus, the basic response (criterion 1 and 2) is correctly reproduced by the model.

In agreement with the literature [13, 11, 14, 15, 16, 17] we see a substantial lowering of the end level of nuclear NF-κB in wild-type cells compared to A20-knockout cells, Fig. 2B. Additionally we find that A20-like proteins are able to adjust the period of nuclear NF-κB oscillations in the range from 0.5 to 2 hours, Fig. 2C.

We have modeled the NF-κB response in A20 knockout cells by removing NF-κB induced A20 mRNA induction in the model \( t_A = 0 \) and were surprised to find that not only is the resting level of the late phase of the NF-κB response increased but also the oscillations period changes compared to wild-type cells, Fig. 2C. In the complete A20 knockout cell simulation the nuclear NF-κB activity is damped and we cannot discuss period and amplitude. We observe that as the A20 feedback gets stronger the period of the appearing oscillations is increased and the amplitude decreased.
Other parameters affecting the period of the nuclear NF-κB oscillations

In Fig. 2 we used $\sigma$ in eq. 7 as a proxy for the A20 feedback strength but in the following we investigate other parameters in the model to see what effect they would have on the period of the oscillations. The following parameters are varied: A20 feedback onto IKK (as in fig. 2), IKK self inactivation, level of TNF induction, A20 transcription rate and A20 constitutive production. They have all been varied in a range around the wild-type parameter value (labelled ‘1’ in fig. 3).

It is seen how both A20 feedback strength ($\sigma$) and A20 transcription rate ($t_A$) are able to increase the period, and likewise IKK self-inactivation, fig. 3. Only the level of TNF has no effect on the period of the nuclear oscillations as the IKK cycle tightly controls the level of protein degradation. Constitutive A20 levels are able to change the period of the oscillations in the presence of NF-κB induced A20, when increased sufficiently.

A20 constitutive level ($p$) is able to bring down the level of IKK activity and the amplitude of the oscillations, but does not influence the period of the oscillations much. Constitutive A20 expression in A20 knockout cells produced damped oscillations with a high steady state but not dependent on the rate of constitutive production.

A20 knockout cell experiments have been performed by Werner et al 2008 [11] and Lee et al 2000 [13]. In ref. [11] a slight change in period can be seen from the wild-type to the knockout cell both in experiments and modeling. The model is fitted to bulk experiments. By using the model presented in ref. [11] it can be shown how the period (time between first and second peak) is modulated by the A20 feedback onto IKK.

Conclusions

Growing evidence indicates that temporal control of NF-κB and the downstream genes are 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 [20] which on a population level appears as damped oscillations. Such temporal control
can allow for selective gene activation [28, 22]. Given that the NF-κB temporal response is of high importance and in single cells is primarily regulated by ‘nested’ negative feedback loops we investigated the role of the A20-like negative feedback.

A specific finding of our investigation suggests that in single cells not only does A20 bring down the resting level of the nuclear oscillations, it can also adjust the period of the oscillations by changing the IKK profile. Thus, A20-like proteins together with IκB allow for independent tuning of frequency through A20-like negative feedbacks (IκBα control the spikiness of the oscillations). Similar characteristics have been observed in ref. [12]. This combination of nested feedback loops covers a wide variety of temporal responses where one can access both sustained oscillations and damped oscillations with low or high frequency.

If the biphasic response – as seen in bulk experiments – is a result of the population average of single cells with oscillating NF-κB, 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 Fig 2B – the second phase should start earlier in A20 knockout cells.

Our findings lead to a clear prediction that in single cells decreasing the coupling between A20-like proteins and IKK should lead to higher frequency oscillations in NF-κB. This can be experimentally tested by knocking down A20-like proteins with siRNA. 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.
Methods

Model Description

\[
\frac{dN_n}{dt} = k_N \frac{(1 - N_n)}{(1 + I_n)} - BI_\alpha \frac{N_n}{(\delta + N_n)} \tag{1}
\]

\[
\frac{dI_{ma}}{dt} = \frac{t_a N^2_n}{(1 + N^2_n)} - \gamma_{ma} I_{ma} \tag{2}
\]

\[
\frac{dI_\alpha}{dt} = I_{ma} - \alpha_\alpha IKK \frac{(1 - N_n)I_\alpha}{1 + I_\alpha} - \gamma_\alpha I_\alpha \tag{3}
\]

\[
\frac{dA20_m}{dt} = t_A N^2_n - \gamma_{Am} A20_m \tag{4}
\]

\[
\frac{dA20}{dt} = p + A20_m - \gamma_A A20 \tag{5}
\]

\[
\frac{dIKK}{dt} = T(1 - IKK - IKK_i) - \mu IKK^2 \tag{6}
\]

\[
\frac{dIKK_i}{dt} = \mu IKK^2 - \beta \frac{IKK_i}{\sigma A20^2 + 1} \tag{7}
\]

The equations are all rescaled, see Supplementary Materials for details of the rescaling and the parameter values. Parameter values and descriptions can be found in table 1.

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 IκB and subsequent export into the cytoplasm. Over the timescales we are interested in there is no significant production or degradation of NF-κB [21]. The mRNA levels of IκBα are regulated through a sigmoidal function of NF-κB, given by \(\frac{N^2_n}{N^2_n + K^2}\). Here we assumed that there is a weak cooperativity in NF-κB activating transcription of IκBα with Hill coefficient two. The model can produce similar dynamics if we replace it with a hill coefficient of 1.

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, $\gamma_\alpha$ and IKK-dependent degradation, $\alpha_\alpha$. The rate of IκBα decay is proportional to both IKK activity and the concentration of complexes formed between IκBα and cytoplasmic NF-κB, $N_c$, $\propto IKK[I\kappa B\alpha : N_c]$. Assuming that reaction rates for complex formation are much faster than the nuclear import/export and IκBα degradation, the concentration of $[I\kappa B\alpha:N_c]$ can be derived to be $(1 - N_\alpha)/(1 + I\kappa B\alpha)$ (see Supplementary Materials for more details). The model has been fitted to reproduce the desired experimental features.

Acknowledgements

We thank Alexander Hoffmann for stimulating discussions.
References


Tables

12
Table 1: Parameters used in the model. *Proportionality factor

<table>
<thead>
<tr>
<th>Process</th>
<th>Name</th>
<th>Value</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF-κB associated rates</td>
<td>$k_N$</td>
<td>$0.2 \text{ min}^{-1}$</td>
<td>Krishna 2006 [21]</td>
</tr>
<tr>
<td>NF-κB nuclear export by IκB</td>
<td>B</td>
<td>$2.4 \cdot 10^{-4} \text{ min}^{-1}$</td>
<td>...</td>
</tr>
<tr>
<td>Conc at half IκB bound to NF-κB</td>
<td>$\delta$</td>
<td>0.0414</td>
<td>...</td>
</tr>
</tbody>
</table>

| IκB associated rates | | | |
| $\rightarrow$ IκBα mRNA (NF-κB) | $t_\alpha$ | $2 \text{ min}^{-2}$ | Fitted, period of 90 min |
| IκBα mRNA $\rightarrow$ IκBα | $\gamma$ | $0.0168 \text{ min}^{-1}$ | Krishna 2006 [21] |
| IκBα $\rightarrow$ deg (IKK mediated) | $\alpha_\alpha$ | $3.6 \text{ min}^{-1}$ | ... |
| IκBα $\rightarrow$ deg | $\gamma_\alpha$ | $0.005 \text{ min}^{-1}$ | ... |

| A20 associated rates | | | |
| $\rightarrow$ A20 mRNA (NF-κB) | $t_A$ | $0.005 \mu M \text{ min}^{-2}$ | Fitted to bulk data [11] |
| $\rightarrow$ A20 mRNA (constitutive) | p | $0.02 \text{ min}^{-1}$ | ... |
| A20 mRNA $\rightarrow$ A20 | $t_L$ | $0.1 \text{ min}^{-1}$ | ... |
| A20 mRNA $\rightarrow$ | $\gamma_{Am}$ | $0.0168 \text{ min}^{-1}$ | Assumed same as for IκB |
| A20 $\rightarrow$ | $\gamma_A$ | $0.01 \text{ min}^{-1}$ | ... |

| IKK associated rates | | | |
| TNF input | T | $1 \text{ min}^{-1}$ | Fitted to experimental ...
| IKK turn off (A20 mediated) | $\sigma$ | $0.25 \mu M^{-2}$ | ...IKK profile [11] |
| IKK turn off (self induced) | $\mu$ | $0.063 \mu M \text{ min}^{-1}$ | ... |
| A20 effect on IKK | $\beta$ | $0.02 \text{ min}^{-1}$ | ... |

Figure 1. Schematic drawing of the nested NF-κB model A) Top: details of the IKK regulation module. Bottom: details of the NF-κB pathway: 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, IκBα and A20-like proteins. The IκB proteins inhibit the NF-κB transcription factor by actively exporting it out of the nucleus. A20-like proteins act up-stream by inactivating IKK. B) Model response, top: NF-κB nuclear/total ratio, bottom: IKK active/total ratio.

Figure 2. A20-like proteins change the period of nuclear NF-κB
Table 2: Rescaled parameters

<table>
<thead>
<tr>
<th>Variable</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$N_n = \frac{N_n}{N_{tot}}$</td>
<td>nuclear NF-κB normalized to total NF-κB</td>
</tr>
<tr>
<td>$I_{α/β} = \frac{I_{α/β}}{K_I}$</td>
<td>IkBs scaled with diss. const. $K_I$ of IkBs binding to NF-κB</td>
</tr>
<tr>
<td>$I_{ma/α/ε} = \frac{I_{ma/α/ε}}{K_I}$</td>
<td>re-defined value of IkB mRNA, $t_{ma/α/ε}$ is the translation rate of IkBα/ε</td>
</tr>
<tr>
<td>$A20 = A20$</td>
<td>A20 protein has not been re-defined</td>
</tr>
<tr>
<td>$A20_m = t_{lA20}A20_m$</td>
<td>re-defined A20 mRNA, $t_{lA20}$ is the A20 translation rate</td>
</tr>
<tr>
<td>$IKK = \frac{IKK}{IKK_{tot}}$</td>
<td>active IKK normalized to the total IKK, $IKK_{tot}$</td>
</tr>
<tr>
<td>$IKK_i = \frac{IKK_i}{IKK_{tot}}$</td>
<td>inactive IKK normalized to the total IKK, $IKK_{tot}$</td>
</tr>
</tbody>
</table>

**Oscillations** A) Schematics of the nested network where the feedback from A20 onto IKK is marked by the yellow arrow. B) Model response of NF-κB nuclear/cytoplasmic ratio and IKK activity for A20Knockout (blue), Wild-type (red) and 56*fold A20 feedback (σ), compared to wild-type (green). C) Schematic drawing of the model with the changes parameter (σ) highlighted. Heatmap recording the period of the nuclear NF-κB oscillations, the steady state and the amplitude of the oscillation. A20-like proteins change the period of nuclear NF-κB oscillations and lowers the steady state level.

**Figure 3.** Change in the period of nuclear NF-κB oscillations due to various different parameters in the model, σ A20 feedback strength, μ IKK self inactivation, TNF is the level of TNF the system is stimulated with. $t_A$ is the transcription rate for A20 mRNA and $p$ is constitutive production of A20 mRNA.
Figure 1:
Figure 2:

Figure 3:
Initial Model for IκBs 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; \( (NI)_n & (NI) \), nuclear and cytoplasmic NF-κB–IκB complex; IKK, IκB kinase.

The equations for IκBε are the same as for IκBα so we just use variable I in our derivations.

The seven-variable model is defined by the equations ([?] supplement)

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

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

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

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

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

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

\[
\frac{d(NI)_n}{dt} = k_{fn}N_nI_n - (k_{bn} + k_{NI_{out}})(NI)_n.
\]

Having the following set of parameters: \( k_{Nin} = 5.4 \text{ min}^{-1}, k_{I_{in}} = 0.018 \text{ min}^{-1}, k_{I_{out}} = 0.012 \text{ min}^{-1}, k_{NI_{out}} = 0.83 \text{ min}^{-1}, k_t = 1.03 \text{ μM}^{-1} \cdot \text{min}^{-1}, k_{tl} = 0.24 \text{ min}^{-1}, k_f = k_{fn} = 30 \text{ μM}^{-1} \cdot \text{min}^{-1}, k_b = k_{bn} = 0.03 \text{ min}^{-1}, \alpha = 1.05 \times \text{IKK min}^{-1}, \gamma_m = 0.017 \text{ min}^{-1}.

Reduced Model for IκBs 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.

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

\[ k_{fn}N_nI_n \approx (k_{bn} + k_{NI_{out}})(NI)_n. \]
Simulations show that these are good approximations. In terms of $I_{n}^{\text{tot}} \equiv I_{n} + (NI)_{n}$ and $N_{n}^{\text{tot}} \equiv N + (NI) = N_{\text{tot}} - N_{n}$, which are slowly varying, we can rewrite the above equations as follows:

\begin{align*}
(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_{n}^{\text{tot}} \frac{N_{n}}{K_{N} + N_{n}}, \\
I_{n} &= I_{n}^{\text{tot}} \frac{K_{N}}{K_{N} + N_{n}},
\end{align*}

where $K_{I} \equiv (k_{b} + \alpha)/k_{f} = 0.035 \ \mu M$ and $K_{N} \equiv (k_{in} + k_{I_{n}^{\text{out}}})/k_{fn} = 0.029 \ \mu M$, using the parameter values above.

Using these expressions, the equations of the seven-variable model reduce to the following four:

\begin{align*}
\frac{dN_{n}}{dt} &= k_{N_{in}}K_{I} \frac{(N_{\text{tot}} - N_{n})}{K_{I} + I} - k_{N_{out}}I_{n}^{\text{tot}}\frac{N_{n}}{K_{N} + N_{n}}, \\
\frac{dI_{m}}{dt} &= k_{I_{m}}N_{n}^{2} - \gamma_{m}I_{m}, \\
\frac{dI}{dt} &= k_{I_{in}}I_{n} - \alpha \frac{(N_{\text{tot}} - N_{n})I}{K_{I} + I} - k_{I_{in}}I + k_{I_{out}}K_{N} \frac{I_{n}^{\text{tot}}}{K_{N} + N_{n}}, \\
\frac{dI_{n}^{\text{tot}}}{dt} &= k_{I_{in}}I - k_{I_{out}}K_{N} \frac{I_{n}^{\text{tot}}}{K_{N} + N_{n}} - k_{N_{out}}I_{n}^{\text{tot}}\frac{N_{n}}{K_{N} + N_{n}}.
\end{align*}

First, we note that the terms $-k_{I_{in}}I$ and $k_{I_{out}}K_{N}I_{n}^{\text{tot}}/K_{N} + N_{n}$ in the $dI/dt$ equation are much smaller than $-\alpha (N_{\text{tot}} - N_{n})I/\alpha K_{I} + I$ and can be neglected as long as IKK is nonzero. Second, simulations reveal that the term $k_{N_{out}}I_{n}^{\text{tot}}/K_{N} + N_{n}$, 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

$$k_{I_{in}}I = k_{I_{out}}K_{N} \frac{I_{n,\text{min}}^{\text{tot}}}{K_{N} + N_{n}} + k_{N_{out}}I_{n,\text{min}}^{\text{tot}}\frac{N_{n}}{K_{N} + N_{n}}.$$
In the regime where $N_n \gg K_n$ this gives

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

Using this we can reduce to a three-variable model

\[
\frac{dN_n}{dt} = k_{\text{in}} K_I \frac{(N_{\text{tot}} - N_n)}{K_I + I} - k_{\text{in}} \frac{IN_n}{\delta + N_n},
\]

\[
\frac{dI_m}{dt} = k_t N_n^2 - \gamma_m I_m,
\]

\[
\frac{dI}{dt} = k_t I_m - \alpha \frac{(N_{\text{tot}} - N_n)I}{K_I + I}.
\]

**Parameters and variables re-scaling**

We start with the following system of equations having extended the three-variable model to include A20 induction and feedback.

\[
\frac{dN_n}{dt} = k_N K_I \frac{(N_{\text{tot}} - N_n)}{(K_I + I)} - B(I_\alpha) \frac{N_n}{(\delta + N_n)} \tag{1}
\]

\[
\frac{dI_\alpha}{dt} = t_{\alpha} I_{m\alpha} - \alpha \gamma K K \frac{(N_{\text{tot}} - N_n)I_\alpha}{K_I + I_\alpha} - \gamma I_\alpha \tag{3}
\]

\[
\frac{dA20_m}{dt} = t_A N_n^2 - \gamma_{A20m} A20_m \tag{4}
\]

\[
\frac{dA20}{dt} = t_{A20} A_m - \gamma_{A20} A20 \tag{5}
\]

\[
\frac{dIKK_i}{dt} = T(IKK_{\text{tot}} - IKK - IKK_i) - \mu K^2 \tag{6}
\]

\[
\frac{dIKK_i}{dt} = \mu I KK^2 - \beta \frac{IKK_i}{\sigma A20^2 + 1} \tag{7}
\]

where $k_N$ is the import of the NF-κB into the nucleus, $t$’s are transcription and translation rates, $N_{\text{tot}}$ is the total NF-κB concentration, $K_I$ is a ratio between IκB - NF-κB complex dissociation and formation, B is a proportionality
factor of the export of nuclear NF-κB, \( \delta \) is the concentration at which half of IκBα is bound in complex with NF-κB and \( p \) is the NF-κB independent transcription of IκBα. Further explanation of the parameters and rates can be found in table 1.

We use the following transformations on the above equations to rescale the model:

\[
N_n \rightarrow N_{tot} N_n \\
N_{tot} \rightarrow K_D N_{tot} \\
I_{ma} \rightarrow \frac{K_t}{t_{ma}} I_{ma} \\
I_\alpha \rightarrow K_I I_\alpha \\
A20_m \rightarrow \frac{1}{t_{A20}} A20_m \\
IKK \rightarrow IKK IKK_{tot} \\
IKK_i \rightarrow IKK_i IKK_{tot}
\]

The final system of equations is:

\[
\frac{dN_n}{dt} = k_N \frac{(1 - N_n)}{(1 + I_\alpha)} - B(I_\alpha) \frac{N_n}{(\delta + N_n)} \\
\frac{dI_{ma}}{dt} = p + t_a \frac{N^2_n}{(1 + N^2_n)} - \gamma_{ma} I_{ma} \\
\frac{dI_\alpha}{dt} = I_{ma} - \alpha_\alpha IKK \frac{(1 - N_n)I_\alpha}{1 + I_\alpha} - \gamma_\alpha I_\alpha \\
\frac{dA20_m}{dt} = t_A N^2_n - \gamma_{Am} A20_m \\
\frac{dA20}{dt} = A20_m - \gamma_AA20 \\
\frac{dIKK}{dt} = T(1 - IKK - IKK_i) - \mu IKK^2 \\
\frac{dIKK_i}{dt} = \mu IKK^2 - \beta \frac{IKK_i}{\sigma A20^2 + 1}
\]
and the scaled parameters are:
\[ N_n = \frac{N_n}{N_{tot}}; \quad I_a = \frac{I_a}{I_K}; \quad I_{ma} = \frac{I_{ma}}{I_{K_I}}; \quad A_m = k_{dA}A_m; \quad IKK = \frac{IKK}{IKK_{tot}}; \quad IKK_i = \frac{IKK_i}{IKK_{tot}}; \quad \delta = \frac{\delta}{N_t} = 0.0414; \quad B = BK_I = 0.014; \quad t_{la} = \frac{t_{la}}{K_I}; \quad p = p\frac{t_{la}}{K_I} = 58.4; \]
\[ t_{a/c} = t_{a}\frac{t_{lc}}{K_I}, t_a = 7300; \quad \alpha'_a = \frac{\alpha_a}{K_D}, \alpha_a = 110; \quad t_A = t_{A20}\frac{t_A}{K_{DA}} = 18; \]
\[ \sigma = \frac{\sigma}{t_{A20}} = 77 \times 10^{-5}; \quad \beta = 5; \quad \mu = K_{tot} = 106; \quad \text{TNF changes from 0.001 to 2.5; } K_{tot} = 6.67 \]
<table>
<thead>
<tr>
<th>Variable</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$N_n = \frac{N_n}{N_{n,tot}}$</td>
<td>nuclear NF-κB normalized to total NF-κB</td>
</tr>
<tr>
<td>$N_{tot} = \frac{N_{tot}}{K_D}$</td>
<td>total nuclear NF-κB normalized to $K_D$</td>
</tr>
<tr>
<td>$I_\alpha = \frac{I_{\alpha}}{K_I}$</td>
<td>free IkBs scaled with dissociation constant $K_I$ of IkBs binding to NF-κB</td>
</tr>
<tr>
<td>$I_{ma} = \frac{t_{ma}}{K_I} I_{ma}$</td>
<td>re-defined value of IkB mRNA, $t_{ma}$ is the translation rate of IkBα</td>
</tr>
<tr>
<td>$A20 = A20$</td>
<td>A20 protein has not been re-defined</td>
</tr>
<tr>
<td>$A20_m = t_{IA20} A20_m$</td>
<td>re-defined A20 mRNA, $t_{IA20}$ is the A20 translation rate</td>
</tr>
<tr>
<td>$IKK = \frac{IKK}{IKK_{tot}}$</td>
<td>active IKK normalized to the total IKK, $IKK_{tot}$</td>
</tr>
<tr>
<td>$IKK_i = \frac{IKK_i}{IKK_{tot}}$</td>
<td>inactive IKK normalized to the total IKK, $IKK_{tot}$</td>
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### Scaled Parameter

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<th>Description</th>
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<tr>
<td>$\delta = \frac{2}{N_I} (\mu M^{-1})$</td>
<td>concentration at which half of the IkBα is bound in complex with NF-κB, normalized to total NF-κB</td>
</tr>
<tr>
<td>$K_D$</td>
<td>Dissociation constant of NF-κB binding to operator site</td>
</tr>
<tr>
<td>$B = B K_I$</td>
<td>proportionality factor of the export of nuclear NF-κB, scaled with the respective translation rates and dissociation constant of NF-κB binding to IkBs, $K_I$</td>
</tr>
<tr>
<td>$K_N = \frac{K_N}{K_I}$</td>
<td>proportionality factor of the import of NF-κB, scaled with the respective translation rates and dissociation constant of NF-κB binding to IkBs, $K_I$</td>
</tr>
<tr>
<td>$p = p \frac{t_{ma}}{K_I}$</td>
<td>constitutive, NF-κB dependent transcription rate of IkBa mRNA, scaled with the respective translation rates and $K_I$</td>
</tr>
<tr>
<td>$t_\alpha = t_{\alpha} \frac{p}{K_I}$</td>
<td>NF-κB dependent transcription rates of IkBs mRNA scaled with $K_I$</td>
</tr>
<tr>
<td>$t_{A20} = t_{IA20} t_A$</td>
<td>A20 transcription rate scaled with A20 translation rate, $k_{tA20}$, and dissociation constant of NF-κB binding to DNA at the operator site controlling A20 promoter</td>
</tr>
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<td>$\gamma_{tma}$</td>
<td>half-life of IkBα mRNA</td>
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<td>$\gamma_\alpha$</td>
<td>half-life of the IkB</td>
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<tr>
<td>$\gamma_{A20m}$</td>
<td>half-life of the A20 mRNA</td>
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<tr>
<td>$\gamma_{A20}$</td>
<td>half-life of the A20</td>
</tr>
<tr>
<td>$\alpha_\alpha = \frac{t_{ma}}{K_I}$</td>
<td>rate constant for IKK dependent degradation scaled with dissociation constant of NF-κB binding to IkBs, $K_I$</td>
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<tr>
<td>$\mu = IKK_{tot} \mu$</td>
<td>rate of IKK self-inactivation scaled with total IKK, $IKK_{tot}$</td>
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<tr>
<td>$\sigma = \sigma$</td>
<td>strength of A20 negative feedback</td>
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Table 1: Rescaling model variables and parameters
At the Core of Gene Regulation: Modeling NFκB•DNA Interactions

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Abbreviations: IκB, Inhibitor of kappa B proteins, NFκB, Nuclear Factor kappa B
Keywords: NFκB, transcription factor, DNA interaction, IκB, stripping,

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Abstract
NFκB is a family of immune stress response transcription factors (TF) whose activity is regulated in a highly dynamic manner and stimulus-specific control is thought to determine which NFκB target genes are induced. The dynamical control of NFκB activation and postinduction repression by IκB negative feedback regulators has been recapitulated in mathematical models (Hoffmann2002, Werner 2005, Lipniacki2004, Krishna2006). However, these models do not include the interaction of NFκB with DNA. Indeed, high affinity interactions of transcription factors with DNA likely impede dynamic control. Unlike previous thermodynamic equilibrium formulations of transcription factor-DNA interactions, which assume that the TF-DNA complex is always in steady state with the nuclear TF concentration, we describe the kinetics of NFκB-DNA complex formation and dissociation, and we distinguish between functional (“target”) and non-functional (“off-target”) binding sites. With this model we explore the role of IκBα’s “stripping” function that facilitates dissociation of NFκB from the DNA (Bergqvist2009). We define the parameter space in which stripping is necessary for faithful stimulus-responsive dynamic control of NFκB-target gene regulation, and we show that a large number of off-target binding sites expand this requirement. Thus our work suggests that negative feedback regulators that facilitate dissociation of TF-DNA complexes render faithful dynamic control more robust not only to variations in the affinity of TF-DNA complexes on target genes, but also to the number of off-target TF-DNA complexes that may otherwise buffer and delay rapid dynamic control.

Introduction
The Nuclear Factor-kappa B, NFκB, transcription factor family are involved in a number of essential biological processes such as immune response, cellular growth and apoptosis, and misregulation has been shown to be implicated in a number of diseases. Nuclear translocation of NF-κB, necessary for its activity, can among others be triggered by tumor necrosis factor (TNF). The transcription factor (TF) has from experiments been found to be in the nucleus for about 70 minutes (Hoffmann2002) in which time it must search and find the DNA site, bind, induce transcription, unbind the DNA and leave the nucleus again. The half-life of the TF:DNA complex might play a critical role for the dynamics of the transcription factor.

NF-κB induces the expression of its own inhibitor proteins, one of which is IκBα. Upon translation in the cytoplasm IκBα translocates to the nucleus where it bind NF-κB. The IκBα-NF-κB complex is exported to the cytoplasm thereby inhibiting the transcription factor and allowing the system for reactivation. Experimental work suggest how IκBα is not only grabbing the free NF-κB in the nucleus but it is capable of actively removing NF-κB from the DNA site, Berqvist 2009. This effect of enhancing the effective off rate of the transcription factor is determined ‘Stripping’. Stripping has earlier been
The half-life of the NF-κB:DNA complex has during the last decades been measured by several groups using different techniques, all giving unique answer to the question of the stability of the TF-DNA complex. *In vitro* measurements by EMSA have been performed by Zabel and Bäuerle 1990 estimating the half-life of the TF-DNA complex to be 45 min and by Phelps et al. in 2000 measuring a half-life of 20 sec of the p50/p65 heterodimer. The first *in vivo* studies done in 2006 by Bosisio et al using FRAP analysis estimated a TF-DNA complex half-life of 1 sec. This wide span from 45 min over 20 sec to 1 sec in the half-life of the TF-DNA complex may be largely due to a difference in the kinds of DNA measured, the different techniques used and even on the NFκB dimer measured. Lately Bergqvist 2009 use Biacore experiments to measure the *in vitro* half-life for NFκB(p50/p65) binding to IgκB sites finding a half-life of the TF-DNA complex of 40 sec at 37°C.

The effect of IκBα actively stripping NFκB from the DNA sites is important when long TF-DNA complex half-life. If the TF-DNA complex half-life is 1 sec or less, as determined by Bosisio 2006 then it does seem surprising if stripping has an important effect for the dynamics of the complex. However, the short half-life measured may apply to off-target DNA interactions and not the DNA interaction with result in promoter activation. Indeed, non-functional DNA interactions, which may be described as interactions with “off-target DNA sites” may be more abundant than functional interactions with “target” DNA binding sites. Common estimates for the amount of NFκB in mammalian cells range around 100,000 molecules. This is contrast to the observation that upon saturating activation of NFκB the expression of about 300 genes is upregulated. Though some target genes require functional NFκB-DNA interactions, it is likely that off-target interactions are much more abundant that target DNA interactions.

The dynamics of the transcription factor NFκB has been modeled a number of times capturing the nuclear localization of NFκB in response to a variety of stimuli (Hoffmann2002, Lipniacki2004, Werner2005, Krishna2006). However, these models does not describe NFκB-DNA interaction and promoter complexes, which is essentially what regulates transcription. The mentioned models are mass action kinetic models driven by reaction rates found by experiments and the tuning of the models is done by fitting the model output to relevant experimental data. Earlier proposed models of TF-DNA interaction are based on equilibrium binding probability considerations (Gerland 2002) but these does not capture the kinetics of the system. We here present a mass action based model of NFκB-DNA interactions to analyze the role of IκBα mediated dissociation of the NFκB-DNA complex.
In this study we consider two types of DNA sites; “target” sites which by interaction with the TF lead to recruitment of the transcription complex and eventually gene transcription, and “off target” sites which does not lead to gene activation. The interaction with “off target” sites is transient with a half-life of 1 sec. The “off target” sites are included in the model to illustrate the large amounts of DNA present in the cell nucleus which the TF can interact with and introduces binding competition for the free NFκB. The biological role of the DNA interaction not leading to gene expression is not yet known but the fact that the TF interact with the huge pool of DNA can be very important for the overall dynamics of the transcription factor. Recent studies have proposed how binding of the TF to the large amounts of off-target DNA is saving the TF from degradation (Burger09). The TF can perform a 3D search for the “target DNA” site by binding off-target DNA and sliding along the DNA in a 1D fashion followed by a release from the DNA and jump to another off-target side. The TF is in this way enhancing the area of search (Sneppen & Zocchi). The amount of off-target sites is not known and has been estimated to be $10^6-10^9$ fold the amount of target DNA has been suggested (Wunderlich08). Another question then arises of what amount of the off-target DNA is accessible for the TF to interact with, and how much is inaccessible and wound up on histones and nucleotides.

The presented model is originally based on Biacore data (Bergqvist09) which by only modeling the target DNA interaction reproduces the data from the experiment nicely. Here we present an extended model including both target and off-target sites in order for the system to be more biologically relevant, figure 1, and to analyze the effect of off-target sites on the dynamics of the TF-DNA complex.

Materials and Methods

(Bergqvist et al 2009) have used Surface Plasmon Resonance (SPR) of Biacore experiments analyzing the role of IκBα in the removal of NFκB from the nucleus and propose that IκBα is actively stripping NFκB from the DNA and thus disrupting the complex. We have built a simple ordinary differential equation (ODE)-based model to address the question of the role of IκBα mediated stripping of the TF from the DNA in the presence on both target and off-target DNA. The model contains the components NFκB, target DNA, off-target DNA, IκBα and their complexes. NFκB can bind to the DNA (k2), unbind the DNA (k-2) and form a complex with IκBα (k1). Additionally IκBα can bind to the NFκB-DNA complex (k3) and dissociate the complex forming a free NFκB-IκBα complex (k4). Likewise NFκB can interact with the off-target DNA, see figure 1.

The on- and off-rate governing the NFκB-DNA complex formation has been determined by Biacore experiments, table 1 (Bergqvist09). Here we are using the rates for the p50/p65 heterodimer as this is the more common and well studied dimer. The IκBα-NFκB complex formation is described by previous
models from which the association rate was found (Werner05). IκBα-NFκB dissociation is set to be zero as it is considered to be very slow and not relevant in the time scale we are looking at. The amount of DNA in the nucleus is large and most probably a large number of sites are accessible for NFκB to interact with. Some of the sites will lead to the recruitment of a promoter and induce gene transcription (target DNA) but other sites will only lead to brief interaction with NFκB and no transcription initiation (off-target DNA), figure 1 (insert box).

In order to achieve a kind of physiological relevant concentration of DNA sites in the model has the number of target DNA sites been estimated to be 1000 as there are roughly 200 genes being transcribed by NFκB and they each are taken to have 5 sites. This has been calculated to 20nM target DNA sites. The amount of off-target DNA is unclear and this concentration is varied in the different simulations. Most likely is the level of off-target DNA at least in the uM range and thereby 100-1000 fold of the target DNA, in agreement with previous estimates (Wunderlich2008). The affinity of the target sites are given by Biacore experiments, with a kD of 5.8nM and a half-life of 100 sec (Bergqvist et al 2009, 25 degrees). The off-target sites have a kD of 580nM and a half-life of 1sec. The on-rate of NFκB binding to the DNA is the same for both DNA types and IκBα can equally bind and strip NFκB from the target and the off-target DNA.

Results

The half-life and stability of the NFκB:DNA complex have been studied by several experimental groups and recently it has been shown that IκBα is able to actively dissociate NFκB from the DNA (Bergqvist09). First, we use a computational model to analyze the role of stripping for the dynamics of the target NFκB-DNA complex in the presence of large amounts of off-target DNA. We do in this first part only aim to present conclusion of the actual DNA interaction and the potential effects from the off-target DNA. Secondly we introduce the DNA interaction module into the full NFκB nuclear response model earlier presented by (Werner 08) to analyze the effect on the target DNA half-life in a larger context.

Stripping is introduced in the model by multiplying a factor to the association reaction of IκBα with the NFκB-DNA complex. Once IκBα is bound to the NFκB-DNA complex the IκBα-NFκB complex will dissociate from the DNA. Stripping has units of μM\(^{-1}\)sec\(^{-1}\) and illustrates the effective NFκB-DNA dissociation rate. A stripping factor of 1μM\(^{-1}\)sec\(^{-1}\) with an IκBα flux of 10nM/sec is equivalent to what (Bergqvist et al. 2009) have determined experimentally by Biacore.

Stripping breaks down the NFκB:DNA complex at high and low concentrations of off-target
First we look at the effect of different amounts of off-target DNA on the dynamics of the target DNA:NFκB complex, **figure 2**, keeping the amount of target DNA at 20nM. **Figure 2A** shows the association of the TF-DNA complex for initial 25 min. At time 0 IkBα is added to the system. The half-life of the complex is the time at which half of the maximum concentration of the complex is left in the system relative to the time at max complex formed. Computationally this has been measured by recording the complex concentration when the system is in equilibrium, right before IkBα is added and measuring the half-life as the time at which the amount of complex is equal half the maximum concentration. The transcription factor – DNA binding is always run to equilibrium before adding IkBα.

The amount of complex formed with target DNA sties is strongly reduced the more off-target DNA present in the system. The off-target DNA is competing for the limited amount of free NFκB thus forming less target complex. This additionally prolongs the half-life of the target DNA complex as NFκB bound to the off-target DNA is able to rebind to the target DNA once released from the off-target sites. This leads to a longer half-life of the target DNA:TF complex in the presence of off-target DNA. **Figure 2B** displays the half-life of the individual simulations in the line plot (**figure 2A**) as a bar graph showing how the half-life is greatly prolonged the more off-target DNA present in the simulation, without active dissociation of the TF:DNA complex.

Adding stripping reveals a large change in the dynamics of the system. The prolonged half-lifes are all dramatically reduced and even shorter half-lifes are found in the presence of larger amounts of off-target DNA, **figure 3 A**. In the presence of stripping, NFκB is captured by IkB already on the DNA site and thereby prevented from self release primarily from off-target DNA and further re-binding to the DNA. Stripping is specifically important at high concentrations of off-target DNA where the half-life of the target complex otherwise is very prolonged.

Simulations are done at different effective values for stripping. A stripping factor of zero is equivalent to not letting IkBα interact with the NFκB-DNA complex and thereby NFκB has to dissociate from the DNA on its own and bind free IkBα for export. Very short half-lifes are found with a stripping factor of 1 or more. Short half-lifes are also seen in the presence of high amounts of off-target DNA as the TF gets trapped on the off-target DNA and the amount of target complex formed is minimal. This small amount of target complex is being dissociated by the ever constant IkBα protein flow. NFκB bound to the off-target DNA is also removed by IkB and is therefore not able to rebind the target DNA as earlier discussed. This leads to the short complex half-life seen in the top right most part of the heat map, **figure 4A**. Long complex half-lifes are seen at high concentrations of off-target DNA and no stripping. The amount of off-target DNA does on the other hand only effects the half-life of the target DNA.
complex vaguely when IκB is stripping NFκB off the DNA, figure 3A-B and figure 4A.

Stripping terminates the NFκB:DNA complex at high and low concentrations of off-target DNA independent on the TF affinity for the target DNA.

The affinity for the DNA site is a measure of how easily the TF is released from the DNA after binding. $K_D$ is given as the ratio: dissociation rate / association rate, and is called the dissociation constant. DNA sites with a high affinity for the TF keep the TF longer on the site as the off-rate is small and so is the $K_D$ of the complex. We are in figure 4B-C changing the amount of off-target DNA vs the $K_D$ of the target TF:DNA complex, having a reference $K_D$ for the Biacore experiments of 5.8nM. This is done without (figure 4B) and with stripping (figure 4C). Interestingly the complex half-life is found to be independent of the affinity of NFκB for the target DNA, if NFκB is being actively removed from the DNA through stripping by IκB, figure 4C. This is seen as a short complex half-life (red) for all concentrations of off-target DNA and all dissociation constants of the target DNA. A short target TF:DNA complex half-life is only found without stripping, at low amounts of off-target DNA and very high dissociation rates (low affinity) of the target DNA, (lower right corner in figure 4B).

**Full model simulations.**

We have now analyzed the role of active IκB mediated dissociation of the TF:DNA complex in a small model where there is no cellular relocation and the endpoint for the transcription factor activity is the binding to IκB. We will next introduce the DNA interaction module in a larger dynamic model of the full NFκB response, earlier presented by (Werner08), where the flux of IκB is part of the model dynamics.

Including the DNA interaction module in the full NFκB model is not completely trivial, figure 5A. The (Werner2008) model has been conducted without DNA interaction and the present rates and interactions are in some way compensating for the lack of DNA. Rates in the model have been set by experiments, calculations and model fit, to allow for a good model reproduction of the data. When directly introducing the DNA interaction module into the model, the dynamics of the response was slowed down and NFκB was now trapped in the nucleus. This is not surprising as the original model is compensating for the lack of DNA interaction in other rates. To include the DNA interaction module in the existing full model we had to carefully decide which parameters to change and fine tune.

Recently, Giogetti10 has predicted that NFκB does not bind cooperatively to the DNA as earlier believed, but with a Hill coefficient of just 1.1. The Hill coefficient has been 3 in the earlier models
(Werner08 and previous). We have, based on this latest finding, chosen to bringing down the Hill coefficient in the model. We have additionally brought down the NFκB induced transcription rate due to the small amounts of target-DNA. A high transcription rate increases the export of NFκB from the nucleus as more IκB proteins are synthesized. All other reaction rates of the full NFκB model are the same as published in (Werner08). The DNA interaction module inserted has the same reaction rates as the small model above, only is the NFκB off-rate from the off-target DNA only 10 times larger than the target DNA and not 100 times as in the small model. Applying these few changes reproduces the data nicely, figure 5C. Other rates as the NFκB: IκB association rate could be changed but we have here chosen to make as few changes as possible, still gaining the over-all same response of the model.

Results:

Elevated basal nuclear NFκB levels.
Including DNA interaction result in elevated basal levels of nuclear NFκB. Bulk experiments using EMSA along with single cell fluorescent microscopy experiments excipit no NFκB in the nucleus before stimulation (Hoffmann02, Werner08, Nelson04, Lee09, Ashall09), indicating no NFκB bound to the DNA in the basal state nor in the nucleus. The basal nuclear NFκB level is highly elevated in simulations including DNA interaction, even when no off-target DNA is present. The transcription factor bind the DNA and the slow off-rate trappes NFκB in the nucleus bound to the DNA. Introducing stripping by the inhibitor protein actively removing the TF from the DNA brings down the basal level of DNA bound NFκB more than 50%, figure 5B.

Off-target DNA
A more realistic model of the biological system is having large amounts if off-target DNA to which the TF can bind shortly but not resulting in gene transcription. Large amounts of off-target DNA slows down the dynamics of the transcription factor (figure5C lower pannel). Only small amount of NFκB is binding the funtional DNA thus producing limited inhibitor protein which then in turn has a hard time exporting the TF from the nucleus (which is bound to the DNA) and thus slowing down the over-all dynamics. Adding stripping brings back the fast dynamics of the NFκB:DNA activity. The profile peaks at 25min and comes back down at 60min. The late phase is damped and goes towards zero by the end of the 6h simulation, figure 5C.

Discussion
NFκB – DNA interaction has been studied for decades in the aim of understading the fundamental underlying dynamics for the transcription factor activity. The time scale for NFκB to induce gene
transcription and leave the nucleus is 70 minutes (Hoffmann02) which is fast and requires a short half-life of the NFκB-DNA complex. A long half-life in the order of 30min or more, would leave the transcription factor on the DNA and thereby not resetting the system with in the time found by experiments. Bergqvist09 have achieved new insight on the half-life of the complex and the stripping function of IκBα. This finding is novel and very important for understanding the dynamics of the transcription factor.

Here we have presented a simple kinetic model which is being used to analyze the effect of stripping in a system of more types of DNA's and with different DNA affinities. We propose that the effect of stripping not only is to maintain the fast dynamics of the transcription factor. Stripping additionally makes the system robust to changes in the level of off-target DNA sites in the nucleus and to the transcription factor affinity for the target DNA. In more physiological terms this is interesting as different cells have different levels of DNA accessible during the cell cycle, cell division and also between cell types. All though large variability in the DNA levels from cell to cell can the dynamics of the TF:DNA complex still be kept the same, when the inhibitor protein is stripping the transcription factor off the DNA. In the same way the transcription factor will interact with many genes with different affinity, but the over all TF:DNA dynamics is kept by stripping, independent on gene affinity.

When including the DNA interaction module in the full NFκB model earlier presented by (Werner08), stripping is found to not only making the dynamics of the NFκB:DNA complex activity faster but more interesting effecting the basal level of the TF:DNA complex and thus the resetting of the system. It has been shown by experiments that there is no free nor bound NFκB in the nucleus in the resting cell. The trapping of the TF by the inhibitor proteins in the cytoplasm inhibits the TF activity but most of all it allows the system for reactivation. If parts of the TF is still bound to the DNA the stimulus specific gene regulations will be less efficient as some gene transcription may potentially always on.

The typology of having a protein actively disrupting the activity of a transcription factor by removing it from the DNA site is most likely not exclusive for the NFκB signaling pathway (e.g. Systems of inducible activator). We do, however, still not know how the stimulus specific gene expression is being preformed and how and if the temporal profile of the nuclear NFκB activity plays a role in gene expression.
References

- Zabel and Bäuerle Cell 61 (1990), **Purified Human IKB Can Rapidly Dissociate the Complex of the NF-κB Transcription Factor with Its Cognate DNA**
- Phelps et al. JBC 275 (2000), **Mechanism of κB DNA binding by Rel/NF-κB dimers**
- Gerland et al. PNAS 99 (2002), **Physical constraints and functional characteristics of**
- Bosisio et al. EMBO 25 (2006), **A hyper-dynamic equilibrium between promoter-bound and nucleoplasmic dimers controls NF-κB-dependent gene activity transcription factor–DNA interaction**
- Bergqvist et al. PNAS (2009), **Kinetic enhancement of NF-κB-DNA dissociation by IκBα**
- Werner et al. Science 309 (2005), **Stimulus Specificity of Gene Expression Programs Determined by Temporal Control of IKK Activity**
- Werner et al. Genes and Development 22 (2008), **Encoding NF-κB temporal control in response to TNF: distinct roles for the negative regulators IκBα and A20**
- Giogetti et al. Molecular Cell 37 (2010), **Noncooperative Interactions between Transcription Factors and Clustered DNA Binding Sites Enable Graded Transcriptional Responses to Environmental Inputs**
- Burger et al. PNAS (2009) **Abduction and asylum in the lives of transcription factors**
- Wunderlich and Mirny. Nature Precedings (2008) *Fundamentally different strategies for transcriptional regulation are revealed by information-theoretical analysis of binding motifs* (Eukaryotic TF have low affinity for sites thus requiring cluster of sites in regulatory regions for specificity. This allows for enhanced combinatorial control.)
- Sneppen, K and Zocchi, G (2005), **Physics in Molecular Biology**, Cambridge University Press
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<td>NFκB – IκB association</td>
<td></td>
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<tr>
<td>k2</td>
<td>NFκB – DNA association</td>
<td>target DNA</td>
<td>1.2 μM⁻¹sec⁻¹</td>
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<tr>
<td>k-2</td>
<td>NFκB – DNA dissociation</td>
<td>target DNA</td>
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</tr>
<tr>
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<td>NFκB – DNA association</td>
<td>off-target DNA</td>
<td>1.2 μM⁻¹sec⁻¹</td>
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<td>k-5</td>
<td>NFκB – DNA dissociation</td>
<td>off-target DNA</td>
<td>0.7 sec⁻¹</td>
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<tr>
<td>k3</td>
<td>IκB – NFκB:DNA association</td>
<td>target DNA</td>
<td>1.0072 μM⁻¹sec⁻¹</td>
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<tr>
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<td>target DNA</td>
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<tr>
<td>k4</td>
<td>IκB:NFκB – DNA dissociation</td>
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<td>IκB – NFκB:DNA association</td>
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<td>k-6</td>
<td>IκB – NFκB:DNA dissociation</td>
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<td>k-7</td>
<td>IκB:NFκB – DNA dissociation</td>
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<td>0.7 sec⁻¹</td>
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Figure Legends

Figure 1. Schematic drawing of the model including off-target DNA. Free NFκB can bind the target DNA (k2), unbind the DNA (k-2) and bind IkB (k1). Additionally, can IkB bind the NFκB-DNA complex (k3) and actively remove NFκB from the DNA (k4). Off-target DNA is added to the model as DNA which NFκB and IkB can interact with in the same way as for the target DNA, only has the off-target DNA a half-life of 1 sec and thereby an off-rate (k-5) which is approx 100 higher than for the target DNA. The complex of NFκB and off-target DNA does not induce gene transcription. Off-target DNA is present in large amounts and introduce competition for NFκB.

Figure 2. Large amount of off-target DNA slows down the dynamics of the target complex. A, Line graph of the amount of target NFκB:DNA complex with different amounts of off-target DNA present in the simulation; 500nM (blue), 2μM (red), 8μM (green) and 3μM (gray). The amount of target DNA is 20nM, the concentration of NfkB is 100nM and the IkB flow is 10nM/sec. No stripping is allowed in the system. Off-target DNA serve as a buffer by binding the TF and when the TF is released from the off-target DNA its free to bind to the target DNA, whereby prolonging the target TF:DNA interaction time. B, Bar graph of the half-life of the target NFκB:DNA in the presence of 500nM, 2μM, 8μM and 3μM off-target DNA measured on the equivalent line graph in (A).

Figure 3. Stripping terminates the NFκB:DNA complex at high and low concentrations of off-target DNA. A, Line graphs of the amount of target NFκB:DNA complex with different amounts of off-target DNA in the presence of stripping. The off-target DNA levels are: 500nM, 2μM, 8μM and 32μM. B, Bar graph of the half-life of the target complex in the presence of 500nM, 2μM, 8μM and 32μM off-target measured on the equivalent line graphs. The half-life is very short for all concentrations of off-target DNA in the presence of stripping. The amount of target-DNA is held constant at 20nM. The half-life of the target DNA is 90sec and the half-life of the off-target DNA is 1sec. The amount of NFκB in the system is 100nM and the IkB flow is 10nM/sec.

Figure 4. Stripping terminates the NFκB:DNA complex at high and low concentrations of off-target DNA and independent on the affinity of the target DNA. Model simulations measuring the half-life of the target-DNA complex at different concentrations of off-target DNA. A: The level of off-target DNA is varied against the effective stripping factor. B-C: The level of off-target DNA is varied against the affinity (kD) of the target-DNA. B: Simulations without stripping reveal very prolonged target-DNA complex half-lifes. These half-lifes are brought down when IkB is stripping NFκB off the DNA, (C). The simulations are performed with a stripping factor of 1μM$^{-1}$sec$^{-1}$, [NFκB] = 100 nM, [IkB]
flux = 10 nMsec⁻¹, [DNA] target = 20nM, k⁻⁵ = 0.7 sec⁻¹

Figure 5. Stripping brings down the basal nuclear NFκB level and reshapes the nuclear NFκB profile. A, The DNA interaction modules has been incerted into the full NFκB model persented by Werner08. We have included DNA interaction in the model and keep all rates the same only changing the hill coefficient from 3 to 1.1, lowering the NFκB induced transcription rate of IκBa and the off rate from the off-target DNA. B, Bar graph of the measured basal concentration of NFκB:DNA target complex, right before stimuli activation. Left hand bars is the basal level when the simulation is run without any off-target DNA, right hand bars is the basal level of the target complex when there is 2μM off-target DNA present. Red: without stripping, blue: with stripping. C, Model simulation resulting time series of the nulear concentration of NFκB bound to target and off-target DNA. There is a total of 20nM target DNA in the system and either no off-target DNA(top) or 2μM off-target DNA (below). The TF can interact with both kinds of DNA but the affinity for the target DNA is 10 times higher the affinity for the off-target DNA. The small amounts of target-DNA in the simulation reduces the NFκB:DNA complex level and a small nuclear NFκB activity is seen, both with and without stripping. Below: The presence of 2μM off-target DNA slows the dynamic profile of nuclear NFκB activity dramatically. Stripping brings the NFκB profile back to its original shape when, measured in Hoffmann02 by EMSA.
Figure 4

Figure 5
Abstract

In many developing tissues neighboring cells enter different developmental pathways, resulting in a fine-grained pattern of different cell states [1]. The most common mechanism that generates such patterns is lateral inhibition, for example through Delta-Notch coupling [2, 3]. In this work we simulate growth of tissues consisting of a hexagonal arrangement of cells laterally inhibiting their neighbors. Mutations are performed by switching the activity of a single node and the effect is quantitative for each node in the lattice. We find that tissue growth by cell division and cell migration tend to produce ordered patterns, whereas lateral growth leads to disordered, patchy patterns. Ordered patterns are very robust to mutations of single cells, and in contrast, mutation of a cell in a disordered tissue can produce a large and widespread perturbation of the pattern. In tissues with complex patterns consisting of ordered and disordered patches, the perturbations spread along the boundaries between patches. The orderness of the patterns depends on the growth speed; if cell division in the tissue occur on a time scale faster than the degradation time, disordered patches will appear. Our work suggests that a careful experimental characterization of the disorder in tissue patterns could directly pinpoint where and how the tissue is susceptible to large-scale damage even from single cell mutations.
Introduction

During development, biological species grow through cell divisions controlled by cell cycles. While most organs exhibit a great variety of forms, tissues are on the other hand close to a two-dimensional layered structure, Fig. 1. The growth of a tissue can thus be well approximated by a layer of cells where the development is either controlled by cell division or by cell migration.

For tissues, we can naturally divide cell-to-cell interactions into two groups: when the cells touch and when they are apart. In the first case, gap junctions may open up and the cells directly exchange signals and molecules. Another possible interaction is through transmembrane proteins [4], like the Delta-Notch receptors [3]. When the cells do not touch, each cell may send out signaling molecules that diffuse around in the tissue until another cell is reached. In the case of gap junctions and transmembrane proteins, each cell obviously communicate with its nearest neighbors and the interactions will in most cases be bi-directed: when a given cell interact with a neighbor, the neighbor interact back with equal 'strength’, Fig. 1 ([5, 6, 7]). In the case of communication through diffusing signaling particles, the interaction with the nearest neighbors is strongest but communications also occur with cells further away, although with decreasing strength. In such cases the interactions may be directed [8].

Figure 1: A). Tissues with hexagonal symmetry from onion epithelium [9] and human colon (right). B). The construction of a tissue on a lattice of hexagonal symmetry with bi-directed interactions. Each black link symbolizes a repression between neighboring cells.

In this paper we model tissue growth on a hexagonal lattice where the
cell interact only with their nearest neighbors. The tissue can grow in two
distinct ways: 1. through cell division where a mother cell gives birth to a
daughter cell in each time step (i.e. cell cycle) of the growth; 2. through cell
migration where silenced cells are migrated towards the tissue and attach
to its boundaries. In general, two neighboring cells will interact with each
other either through activation or repression. The last case, when two cells
repress each other, usually results in switch-like behavior [10] where a cell
is either in a high/active or in a low/silenced state. Thus a tissue where
all neighboring cells interact through bidirected coupling on a regular lattice
will results in an intricate state where active and silenced states form in a
complicated pattern. This type of idealized tissue is shown schematically in
Fig. 1.

Previously, other papers have studied coupled repressilators to investigate
quorum sensing [11] and to describe cell-to-cell communication [12]. Recently,
Jensen et al [8] proposed a model of cell-to-cell interactions in a planar tissue
where local repressilators were placed on a hexagonal lattice. In ideal situ-
ations, tissues sometime display hexagonal or near-hexagonal structure (as
indicated in Fig. 1), e.g. in hepatic or retinal tissue [13, 14, 15], especially
in planar tissues because of close packing of cells. The repressor-lattice pre-
sented in [8] consisted of directed interactions leading to oscillatory states of
individual cells sometimes resulting in a synchronized oscillatory pattern of
the entire lattice.

Methods

The model we propose here consists of a tissue of cells each labeled by a node
number \((m, n)\). This cell is repressed by the six neighboring cells (see Fig.
1) which is represented by an interaction term \(F_{\text{int}}\), leading to a dynamical
equation for its activity \(x_{m,n}\):

\[
\frac{dx_{m,n}}{dt} = c - \gamma x_{m,n} + \alpha F_{\text{int}} \tag{1}
\]

We consider here a mutual repression on multiplicative form:

\[
F_{\text{int}} = \frac{1}{1 + \left(\frac{x_{m+1,n}}{K}\right)^h} \cdot \frac{1}{1 + \left(\frac{x_{m-1,n+1}}{K}\right)^h} \cdot \frac{1}{1 + \left(\frac{x_{m+1,n+1}}{K}\right)^h} \cdot \frac{1}{1 + \left(\frac{x_{m-1,n-1}}{K}\right)^h} \cdot \frac{1}{1 + \left(\frac{x_{m+1,n}}{K}\right)^h} \cdot \frac{1}{1 + \left(\frac{x_{m-1,n}}{K}\right)^h} \tag{2}
\]
The repression of one cell to another is expressed through a standard Michaelis-Menten term. The parameter \( c \) determines a constitutive production, \( \gamma \) a degradation rate and \( \alpha \) the strength of the repression by neighboring cells. Further, \( K \) is the dissociation constant of the binding complex whereas \( h \) is the Hill coefficient measuring its cooperativity. For simplicity we assign the same coupling strength \( \alpha \) to all cell to cell interactions in the lattice. A typical set of parameter values is: \( c = 0.1, \gamma = 1, \alpha = 2, h = 3, K = 1 \).

In all simulations presented in this paper we apply open boundary conditions meaning that all cells outside the tissue are silenced, i.e. \( x_{m,n} = 0 \) adjacent to boundary cells. This is in contrast to the studies of directed interactions in Ref. [8] where periodic boundary conditions were applied to obtain oscillating solutions.

We explore four different ways of growing a tissue.

1. Through **cell division** where the system is initiated by 4 cells in random initial states (ranging from silenced to active). Using the dynamical equations, the cell cycle process proceeds in two steps: the cells are dynamically equilibrated after which one of the cells (chosen randomly) is doubled. The new daughter cell has the same level of activity as the mother cell. Further, the new cell pushes a row of existing cells in a random direction. The tissue with one new cell is now equilibrated after which a new cell is doubled, and so on, Fig. 2A.

2. Through **cell migration** where new cells arrive only at the boundaries of the tissue. All new cells are silenced/inactive. In each step, a new layer is added adjacent to all existing tissue cells after which the tissue is dynamically equilibrated by the dynamical equations, Fig. 2B.

3. Through **lateral growth**. Here, new cells are added layer by layer in one direction only, starting with one row of cells. The new row of daughter cells is obtained through cell division from the existing mother cells just below, Fig. 2C. After the growth of a new layer, the tissue is equilibrated.

4. Through a **random configuration of cells** in silenced and active states. Here there is no growth as the tissue keeps the size of the initial configuration. After initiation, the entire tissue is equilibrated into the final state.
Figure 2: Three different growth mechanisms for cell tissues on hexagonal lattices. A). Cell division: A random cell in the tissue is doubled in each time step. The daughter cell is born in the same state as the mother cell and existing cells are pushed outwards (in a random direction) by the newly born cell. After each cell cycle the entire tissue is equilibrated by the dynamical equations (). B). Cell migration: Cells are in each time step migrated onto the entire boundary of the tissue. All newly arriving cells are in the silenced state. The tissue is equilibrated between each growth step. C). Lateral growth: the tissue is grown only along one side through cell divisions, where each daughter cell is born in the same state as the mother. Between each addition of a new layer, the entire tissue is equilibrated. The parameter values were: $c = 0.1$, $\gamma = 1$, $\alpha = 2$, $hill = 3$, $K = 1$. 
Figure 3: Tissue growth under cell division where in each cell cycle, a random cell is chosen and multiplied such that the daughter cell is born in the same state as the mother cell. The new cell pushes existing states in a random direction. The cell a graded from black (active cell) down to light gray (silenced cell). Note that the center of the tissue becomes completely ordered where an active cell is surrounded by six silenced cells.

The four ways of building the tissue produce quite different patterns of silenced vs active cells. Fig. 3 shows a tissue grown through cell division method 1), where one cell is doubled in each time steps. Note that after 291 divisions, Fig. 3C, the center of the tissue is completely ordered with every active cell (black) surrounded by six silenced cells (light gray), and conversely each silenced cell will have three active and three silenced neighbors. The boundary is naturally still somewhat disordered but as the tissue expands, the pattern becomes completely ordered. It is easy to understand that an ordered state, with each active cell surrounded by six silenced cells is an 'equilibrium' state of the dynamical equations because the active cell in the middle respects the equations through repressions of the six neighbors.

Similarly, tissues built by cell migration, method 2), also evolve into completely ordered pattern. This again forms a regular, symmetric pattern where one in any of the symmetry direction observes periodic arrays of one active cell followed two silenced cells Fig. 4B. Lateral growth results in random patches of disordered and ordered tissue Fig. 4C, whereas the random initial conditions produce a very disordered tissue Fig. 4D.

A central theme of our paper is to study the robustness to mutations of the different tissue patterns described above. A point mutation is performed
Figure 4: The accumulated effects of point mutations in the entire tissue. Starting from four different equilibrated tissues grown by cell division (A), cell migration (B), lateral growth (C) and random initial conditions (D), respectively, each cell of the tissue is point mutated by the following rule: silenced $\rightarrow$ active or active $\rightarrow$ silenced. The mutated cell is kept in the state after mutation. The color scheme indicate the accumulated change over the entire tissue due to one single point mutation. We note that the effects of mutations in the ordered tissue (B) are small and these are most robust to mutations. The tissues with disordered patches are very sensitive to mutations on the defect lines separating these two phases. This is also apparent in the randomly grown tissues where the larger effect occur all along the defect boundaries.
by switching the activity of a single cell from silenced to active or from active to silenced (depending of the state the cell has before mutation). To record the effects of mutations we perform this type of point mutations, one after the other, in every cell of the tissue, with the identical equilibrated state as the starting point. The recording of the effects of the mutations is performed in mainly two ways by measuring:

- The total change in cell activities after a single point mutation, measured in the entire tissue
- The number of cells whose activity is affected more than 5% after a single point mutation

Fig. 5 shows an example of a point mutation performed on an equilibrated tissue with a stripe of disorder (left panel). On the boundary to the disordered patch (given by the ragged line) a cell is mutated from a silenced to an active state, middle panel. Fig. 5 right panel show the total distortion due to the mutation where the state of 10 cells are affected more than 5%. The color indicates which cells are affected the most (red) and which are affected the least (blue). The same procedure is performed in each cell of the tissue.

**Results**

Fig. 4 shows the combined results of mutations in the four different tissue we consider: tissues grown by cell division (Fig.4A), by cell migration (Fig. 4B), lateral growth (Fig. 4C) and random initial conditions (Fig. 4D). The main conclusion are two-fold and are very clear from this figure: 1) *The ordered tissues are more robust against mutations than the disordered*. 2) *The most pronounced effects of mutations occur at the boundaries between the ordered and disordered patches.*

The tissue in Fig. 4A is completely ordered before mutations. We observe that the effects of mutations are very small and only for a few cells close to the boundaries one observes a detectable effect – definitely due to a boundary effects. Tissue Fig. 4B has well established defect lines between ordered and disordered patches. Clearly, the tissue is less robust against mutations exactly along this line. The same picture is seen also for the last tissue, Fig. 4C. Along the defect lines, separating ordered from disordered tissues, the
Figure 5: A point mutation in a tissue grown through cell division. On the
defect boundary between the ordered and disordered tissue (left panel), a
silenced state is mutated into an active state (middle panel). The rightmost
panel shows the effect of the mutation, where red symbolizes the largest effect
and blue the smallest. The total number of cells that are affected more than
5% by the mutation is 10 (all colored).

effects of mutations are predominantly more pronounced than within ordered
patches.

In order to make more quantitative statements, we show in Table 1 the
average number of cells affected (i.e. more than 5 %) for a point mutation:
silenced $\rightarrow$ active or active $\rightarrow$ silenced. The first coulomb are averages due
to mutations over the entire tissues (except close to boundaries to avoid
boundary effects) where the first line shows measurements for all cells that
were silenced before the mutations while the second line shows measurements
for all cells that were active before mutations. We note that the ordered
tissue grown by cell migration is most robust to mutations. In particular,
for the class of mutations active $\rightarrow$ silenced the ordered tissue is extremely
robust. For the tissue with disordered patches, we observe that the most
pronounced effect of mutations occur when the point mutations takes place
within a distance of two cells from the defects (see Fig.5). This is the case
both for tissue grown by cell division and by lateral growth. An exception is
the completely random tissue but here defects are so dense in the tissue that
it is difficult to distinguish ordered from disordered patches.

Fig. 6 shows a growth series, similar to the one in Fig. 3, with the
difference that mutations are now made during growth. For a specific cell in
5 % of the cell division, the cell is mutated (and kept) into either a silenced
(yellow) or active state (green) state. Note that this creates a tissue with
strongly disordered patches. As the tissue expands, such mutations lead to
a rather disordered tissue.

Figure 6: Mutations during growth of a tissue similar to in Fig. 3. In 5 % of the cell division, where the cell is mutated (and kept) into either a silenced (yellow) or active state (green) state. Note that this creates a tissue with strongly disordered patches.

During growth via cell divisions, the tissue is equilibrated through the dynamical equations after each cell cycle. A natural time scale of the growth is set by the degradation time \( \tau_d = 1/\gamma \). In general we let tissues equilibrate over time period \( T_e \) that are several orders of magnitude larger than the degradation time, resulting in ordered tissues. In order to investigate how variations in equilibration time influences the resulting tissue patterns, we show in Fig. 7 three stages of the growth with an enforced speed where \( T_e \approx \tau_d \). Clearly, this type of fast growth leads to a tissue that is disordered with patches where the equilibrium state is not reached.

**Discussion**

The main results of the paper fall in two categories: the first deals with order and disorder in the growth of tissues, the second with stability of tissues due to mutations. We have presented a model for tissues growth during development with an underlying hexagonal symmetry. This is a natural ordering of cells that are closed packed in a single layer (see Fig. 1) and we have assumed that all neighboring cells repress each other. Seen from a biological viewpoint, the most natural way to grow a tissue is through cell
Figure 7: A tissue grown with enforced speed. The equilibration time $T_e$ between each cell cycle is of the size of the degradation time $\tau_d = 1/\gamma$ and is thus much shorter than the equilibration time used in Fig. 3. We note that both disordered and ordered patches now appear side by side in the tissue.

divisions. We find that this invariably leads to ordered tissues. Cell migration defines an alternative way of growth which also leads to ordered tissues in the bulk. In both cases, some disorder is observed close to the boundaries of the tissues. Lateral growth, where an entire row of cells is integrated into the tissue in each cell cycle on the other hand leads to quite disordered patterns with default lines penetrating the tissue.

Introducing mutations, we observe that the ordered tissues are much more robust and stable towards mutations than are the disordered tissues. Mutations in the ordered tissues only lead to localized disorder with almost no effect. Contrary to this, in the disordered tissues, in particular when mutations are performed very close to the default lines, mutations have more pronounced effects and can lead to patches of sick cells. The biological implications of these observations are quite fundamental: tissues which exhibit patches of disordered cell structure might be much more accessible to mutations and thus to a possible development of illnesses, eventually leading to cancer. We have furthermore observed that disordered tissue might also appear during enforced speed in the development. This indicate that in order to achieve biologically healthy stasis for a tissue, the newly formed cells need reasonable time to be balanced and equilibrating with the surrounding cells.
Table 1: Average number of cells which are affected more than 5% due to a point mutation of either a silenced cell to active, or an active cell to silenced. The upper two-line column is for the entire tissue. The middle column is for cells which a distance of two cells from a defect. The lower column is for cells that are further away that two cells from a defect. Only cells within a distance of 3 cells form the boundary are included in the 50x50 tissue to avoid boundary effects. A tissue built by migration has no defects and thus no counts in the 4 lower rows. The average also include cells that have less than 5% change (contribute a zero and thereby lowering the average).
References


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Network Analysis of Human Interactions: Revealing Differences in Preterm vs. Full Term Infant-Mother Interactions.

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Abstract (225 words)

Understanding and predicting human behaviour has been a central question in the history of mankind. Recently, interest turned to quantitative analysis of human activities using mathematical models and network tools, addressing temporal and structural features of human communication. In this paper we apply network analysis tools to identify patterns in mother-infant interactions. Previously, a remarkable number of studies reported that interaction of mothers with preterm infants show differential characteristics compared to that of mothers with full-term infants. However, observations and explanations concerning the underlying mechanisms are inconsistent. We present findings of a comparative study of the early mother-infant relationship. Preterm and full-term babies’ and mothers’ behaviours were observed in dyadic situations and coded micro-analytically. We attempt to eliminate inconsistency by an in-depth analysis of the coded data through formation of complex interaction networks and transition patterns between combined infant/mother states. Comparison of preterm and full-term dyads shows that interaction of preterm dyads are often less harmonious, which may result in a developmental lag. We find that mothers of preterm infants are periodically at two extremes of maladaptive behaviour: they are either intrusive or disengaged. The pairing of an oversensitive infant with an intrusive/disengaged mother may place the infant at risk for later emotional, cognitive and behavioural disturbance. Our results call for invention of intervention techniques which can protect development of preterm infants when such maternal behaviour is observed.

Newborn infants are considered preterm if born earlier than 37 weeks of gestation and/or with birth weights less than 2500 grams. Preterm infants are more likely to experience a developmental delay than their full-term peers. Prematurity is not an illness and does not unconditionally causes a developmental delay; however, preterm babies are at risk of impaired cognitive and social development. A preterm infant’s developmental prospect depends on risk- and protective factors. Attempts to apply perinatal risk scales for understanding and predicting the outcome of their development have not been successful. Subsequent attention turned to environmental factors such as socio-economic status and the quality of life. Because the explanatory power of environmental factors was also found to be weak, the research focus turned toward mother-infant interactions. Caregiver-infant interactions have been found to contribute to the developmental outcome through complex transactions between the infant characteristics and the maternal behaviours.

Premature birth creates an atypical condition for mother-infant interactions, and the weaker self-regulation of the preterm baby requires a higher degree of adaptation from the mother. A growing number of evidence suggests that maternal behaviours toward premature babies may have differential characteristics, which are either adaptive or maladaptive in light of the preterm baby’s atypical needs. Reported data on the behaviour of preterm mothers are inconsistent.
Various reasons may account for the inconsistency, e.g. the degree of immaturity, perinatal complications in the infant, preparedness of the mother, support available to the mother, the infant’s age at the observation, and the context of interaction\(^4,16\).

In addition, there are distinct ways of how data are derived from the observed events. The majority of studies on mother-infant interactions used global rating scales\(^17,18\), which may be helpful in detecting certain features of the interaction. However, global rating scales miss to catch the sequences of behaviours. Micro-analytic (frame by frame) coding systems are suitable for recording bidirectional transactions\(^19,20\). In this study connected networks were constructed from micro-analytically coded infant-mother interactions in order to capture key characteristics and differences in preterm and full-term infant-mother interactions. Thirty preterm and 42 full-term infant-mother dyads were videotaped in play situation (see Supplementary Materials for details). Behaviours of the mother and infant were coded using a mutually exclusive and exhaustive micro-analytic category system. Preterm and full-term mother-infant interaction networks were visualized separately using Cytoscape\(^21\), where each node represents a combination of infant and maternal behaviours (termed “state”) and the links between the nodes represent transitions between states (Figure 1). In these networks each transition was quantified by counting the number of occurrences of the particular transition in a given group and normalizing it to the total number of transitions observed in the micro-analytically coded data series in that group. The value obtained, termed “transition rate”, can be considered a percentage which lies between 0 and 5%. The structures of the networks obtained are not modular\(^22\), very few sub-network structures were found. The optimal state (infant plays/mother follows) is highly connected in both the preterm and full term groups, therefore we placed it in the centre in Figure 1. Human behaviour is often considered to be random and Poisson distributed\(^23\). Analysis of the transition probabilities between different states shows non-Poisson statistics with a few transitions occurring with high frequency (Figure 2, highlighted in Figures 1A and 1B). While most of the transitions in the infant-mother behaviour networks seem to be unsystematic, the existence and topology of the high-frequency transitions in the networks suggest that there are regular patterns in the interaction.

In order to compare the interaction patterns of full-term and preterm dyads, the transition networks were subtracted from each other. In this way the distinctive transitions become visible. Figure 1C represents the transitions which have more than 0.5% difference in the transition rates between the groups. The subtracted transition networks suggest that there are potential distinctive paths in the system, therefore in the coded data we recorded the sequences of transitions before distinctive transitions, and calculated how frequent a given state appears at a specific position in the sequences. In Figure 3 we show the possible states preceding three transitions: 1-12→1-11, 4-15→4-11, and 6-18→6-15. The 1-12→1-11 is an optimal transition, which occurs in the full-term group more often than in the preterm group, and is often periodic. In this case the infant plays based on his/her own idea, while the mother alternates between following and enriching his/her activity. It means that the mother gives the control to the infant for choosing what to play, but stays involved in the interaction and helps maintain the infant’s
attention by occasionally enriching, elaborating his/her ideas. This maternal behaviour is favourable in four aspects: (1) helps the infant to develop focused attention by staying longer in a certain activity, (2) teaches the infant new ways of playing with one or the other toy, therefore facilitates acquiring knowledge and skills, (3) allows the infant to experience that he is an able-to-act agent (4) provides mutual happiness and satisfaction in the interaction. Interestingly, during the sequence preceding the 1-12→1-11 transition mothers of full-term infants are predominantly in states 11 (follow), 12 (enrich), or 20 (handle toy), while mothers of preterm infants often can be found in state 14 (command), controlling the infant’s activity. The 3-15→4-15 transition (mother ‘directs attention’, infant switches from ‘obey’ to ‘cooperate’, i.e. plays according to the mother’s idea happily) is more than twice as frequent in the full-term group then in the preterm group. In this transition the infant turns the interaction more harmonious. The typical subsequent transition, the mother stops directing the infant (4-15→4-11) occurs about twice more frequent in the full-term group. The 6-15↔6-18 transitions (mother directs/neglects infant while infant neglects mother) happen very rarely (only 4 times) in the full-term group (6-18→6-15 in case of 1 infant, and 6-15→6-18 in case of 3 infants), and infants started to play 8 to 15 seconds after the transition, leading to a harmonious state (1-11) eventually (mean=29 s, SD=25 s). In the preterm group the 6-15↔6-18 transitions occurred more frequently ($\chi^2<0.05$), we found at least one transition in 30% of mother-preterm infant observations, and in 23% of the cases we observed more than one transition. In these transitions the mothers of preterms respond to the neglecting behaviour of the infant by directing the infant’s attention or withdrawing themselves from the interaction (neglecting the infant), often alternating these behaviours. Interestingly, in the preterm group the 6-18→6-15 transition only led to harmonious play (1-11 or 1-12) within ~2 minutes (117 s) or longer (mean=248 s, SD=91 s), and in 21% of the cases the interaction did never return harmonious after this transition. In case of the 6-15→6-18 transition we found only one case when harmonious play (1-11) was reached in a short time (10 seconds), which represent about 5% of the transitions. Our data shows that in the preterm group the 6-15→6-18 transition is one of the most unsuccessful maternal transitions from the 6-15 state. The different reaction observed in the two groups could be explained by the more mature nervous system of full-term infants, which may make them less sensitive to their mother’s withdrawing from the interaction.

We also created separate networks for the infant and mother data sequences. In Figure 4 we present the differences of preterm and full-term infant and maternal behavioural networks. The results suggest that interaction of full-term infants and their mothers are more focused and harmonious. Full-term infants spend significantly more time playing based on their own ideas than their preterm peers, and transitions are more frequent between playing, cooperating, exploring and obeying. The major difference in the maternal behaviours is that the transition pattern of mothers of full-term infants is focused on three states, following and enriching the infant’s activity and directing his/her attention to new ideas. These transitions are more frequent than in the case of mothers of preterm infants, whose transition pattern is more evenly spread.
Our results also show that mothers of preterms more often switch from pushing their ideas (direct attention) to either using physical force (15→13) or neglecting (15→18) behaviours. As compared to full-terms, mothers of preterms are more prone to use a kind of power politics. They seem to insist on their ideas more intensively, and accept the infant’s different wishes less often. Several reports\textsuperscript{24-27} have suggested that mothers who gave birth prematurely feel guilty because of the shortened intrauterine period and they may try to compensate the infant for it. The more controlling and attention-directive behaviour can be an attempt to re-mediate their self-image. The paradox is that the more the mother tries to force her own idea over the infant’s one, the fewer positive answers she gets from the infant because preterm infants neglect the mother’s controlling behaviour. Subsequently, the mother disengages, establishing a \textit{circulus vitiosus}. Nevertheless, in the full-term group both partners stay involved and they share a mutual focus of attention almost all the time.

Our results highlight important differences in the interactions of the two groups. However, the preterm group shows a large diversity in the nature of mother-infant interactions. Therefore following the infant’s development in our longitudinal study may be able to answer what are the long-term consequences of maladaptive maternal behaviour.

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References


**Supplementary Information** is linked to the online version of the paper at www.nature.com/nature.
**Figure 1:** Interaction networks of the combined mother-infant behavioural transitions in full-term (left panel) and preterm (middle panel). The nodes (filled red circles) represent combined mother-infant behavioural states and their size is a measure for the average time spent in the combined state. The combined states are attributed an infant state labelled from 1 to 6 and a mother state, labelled from 11 to 20. Infant states are indicated in the outer ring (grey bars), and mother states are shown sequentially for each infant state (a few numbers are shown for easier identification of states). The different mother states are listed to the right. The links between the nodes indicate possible transitions from one combined mother-infant state to another. The width and colour of the links change according to the transition rates (in percentage), normalized within the group of either full-term or preterm (see the colour scale). Transitions with probability less than 1% are coloured green. **Right:** The difference between the transition networks of the full-term and the preterm mother-infant states. Red arrows indicate the full-term transitions with a difference of 0.5 or more to the preterm. Blue arrows show preterm transitions which are 0.5 or more frequent than in their full-term peers. The width of the links scale with the values of the transition differences and the node sizes scale with the difference in time spent in the states.
Figure 2: Distribution of the transition rates in full-term and preterm combined behavioural networks. The transition rates are measured as the normalized percentage within each group. The probability density function of transition rates are plotted on double-logarithmic scales indicating an underlying scale free network with scaling exponent -2. The full drawn curve shows an associated random Poisson distribution derived around the mean of the transitions rates from both data sets.
Figure 3: Transition paths in the full-term and preterm groups preceding transitions 1-12→1-11, 4-15→4-11, and 6-18→6-15. Transition rates (TR) are indicated. The large triangles show the distribution of states preceding the above transitions, and the most frequent state occurring in the previous steps in the sequence (coloured red or blue if there is one). The baselines of the large triangles are divided proportionally to the occurrences of the states. Only states occurring with ≥ 5% frequency are shown. The 6-18→6-15 transition sequence in the full term group represents a single event.
Figure 4: Differences between the full-term and the preterm transition networks of the mothers (left) and infants (right), treated separately. Each node represents either an infant or a mother behavioural state with numbers referring to the states listed in Figure 1. The size of the nodes is a measure for the differences between the average times spent in the states. Dark grey nodes indicate states in which preterm infants/mothers spent the longest time whereas white nodes indicate states where full-term infants/mothers spent the longest time. The arrows between the nodes represent transitions between different states, and the width of the arrows is a measure for the difference between the normalized transition rates in the two groups. Red arrows are the transitions which occur more frequently in the full-term group while blue arrows are transitions which occur more frequently in the preterm group. To highlight the transitions with large differences, all transitions rates below a threshold of 1% are coloured grey.
Methods

Design
The data presented and analyzed in this paper are from a prospective longitudinal study aiming at detecting the determinants of developmental outcome of preterm children.

Subjects
72 infants and their mothers participated in the study. Thirty infants born preterm, at 28 - 33 weeks of gestation (mean GA 30.9 weeks, SD 1.5 weeks), with birth weights of 800 - 1990 grams (mean BW 1437 grams, SD 260 grams). The children possessed no congenital abnormalities or obvious sensory deficits, and their perinatal course was free of severe complications. Their risk scores on the Parmelee Obstetric and Postnatal Complication Scales\(^1\) ranged between 6-17 (mean 10.4, SD 2.9), and they were regarded by the neonatologists as low- to moderate risk babies. The male/female ratio was 50/50 % (none of the perinatal variables was related to gender).

The gestational age range for the preterm infants was chosen with certain considerations in mind. After 28 weeks of gestation, with good perinatal care and if the organism is otherwise healthy, the degree of maturation enables the central nervous system to adapt the vital autonomic processes to the extrauterine conditions without life-threatening difficulties. On the other hand, it is an extremely important period in the development of alertness and state regulation, and in this respect these preterms are expected to be still markedly different from the full-term neonates\(^2\). The ages in the preterm infants’ were corrected according to their expected birthday.

The comparison group of 42 healthy full-term infants (GA > 37 weeks, mean BW 3421 g, SD 374.3 g, range 2650-4350 g, 52 % boys, 48 % girls) and their mothers were selected from the subjects of the Budapest Parent-Infant Study\(^3\). The mean age of the mothers was 28.3 years in the preterm group (range: 20-42), and 26.6 years in the comparison group (range: 19-34).

The two groups were matched on demographic variables, yet on one variable matching was not fully successful. Mothers of full-term babies had higher levels of education.

Procedure
Mother-infant dyads were observed at the infant’s age of 12 months in a play situation at home. Observational sessions were recorded on video by a female researcher. Each visit began with a familiarization period, lasting about 10 minutes. After that the mother was asked to play with her child as she ordinarily would and to disregard the researcher’s presence as much as possible.

Data collection
The categories of the infant and the maternal behaviors were the following:

Infant: 1: plays (plays with a toy of his/her interest); 2: explores (searches for/approaches new toy); 3: obeys (complies with the mother’s wishes without expressions of positive emotion); 4: cooperates (happily accepts and follows the mother’s idea); 5: disobeys (actively opposes against the mother’s idea/command); 6: neglects (ignores mother or her ideas, does not comply with the mother’s command but does not oppose explicitly); 7: passive (is not involved in any activity); 8: other (none of the above categories)

Maternal: 10: other (none of the categories below); 11: follows (follows the infant’s idea, she adapts herself to the infant, they focus on the same thing, mother is involved); 12: enriches (enriches the infant’s play with her own idea, but does not change toy/game, elaborates the infant’s play, shows a new aspect how to use a toy); 13: physically forces (physically forces or prevents the infant of doing something); 14: commands (demands the infant to do something); 15: directs attention (intrusively directs the infant’s attention. She insists on her own idea, irrespective of the infant’s involvement in doing something else); 16: interrupts (interrupts the infant’s play activity with anything else but directing the infant’s attention to another toy, e.g. cleans the nose, adjusts clothes of the infant etc.); 17: passive (not doing anything and being uninvolved); 18: neglects (not playing with the infant, and actively doing something else); 19: insensitive (any behavior not satisfying the infant’s obvious need, expressing disappointment about the infant’s behavior, or expressing developmentally unreachable expectation towards the infant); 20: manipulates toy (not playing but manipulating the toy to promote the infant’s activity, e.g. assembling a toy).

Interrater reliability was established by coding 14% of the sample by two independent coders. Cohen’s kappa was based on whether the coders agreed with the behavior category within 2 seconds, kappa= 0.82.

Network analysis

The network of behavioral transitions was extracted from the original data (using custom scripts in MatLab) by recording every change in behavior in either the infant state, the mother state or in the combined mother-infant behavioral state. The ‘other’ states (8 and 10) were omitted from the analysis because they cannot be linked to a specific behavior. The behavioral states appear as the nodes of the network and transitions between the states are the links between the nodes. In this way we are eliminating time and only looking at the possible transitions between behavioral states. This analysis was performed in the entire group of either full term or preterm infants. The importance (rate) of one specific transition was found by dividing the number of times this specific transition was recorded in the group by the total number of transitions occurring within the group. In this way,
the transition rates are normalized to both the number of infants in each group, and the length of the individual recordings. The transition rates were exported as a Cytoscape compatible file and used for the edges/links in the network. The average time spent in a mother, an infant, or a combined mother-infant state was exported as a Cytoscape compatible file and used for to determine the size of the nodes in the network.

Subtracted interaction networks have been generated by subtracting the transition rate of a given preterm transition from the same transition in the full term network, and thus obtaining the difference between the two groups. Positive differences above 0.5 and negative differences below -0.5 were exported as Cytoscape compatible files and used to generate the edges/link of the subtracted network. In addition, the time spent in each state was subtracted between the two groups and exported for Cytoscape and used for the scaling of node size. The differences have not been normalized.

**Distribution of the importance of links**

The distributions of the importance of the links in the network were plotted as a histogram in MatLab, using a binning of 0.05. In order to compare the distribution with a random process, a corresponding Poission distribution has been constructed around the average transition weight (full term and the preterm) using MatLab. The probability density function has been derived by logarithmic binning of the transitions weights after normalizing the count to the width of the individual bins, plotted on double logarithmic scales.

**Path analysis**

Distinctive transitions were collected from the subtracted transition networks of the combined mother-infant states. We have focused on the transitions leading up to an important transition by recording the states just before this transition. Of all the states leading up to a specific behavioral transition, we have recorded how many times one state appears. From this we can generate the rates (in percentage of all states) leading up to the specific transition. This analysis has performed for 1-3 steps before the actual transitions.

**Statistical significance**

The dominant transition between 1-11 and 1-12 was tested against randomized networks to get a measure of the significance of the weight of this transition. We have from the original interaction
networks of the data generated 5000 random full term and 5000 random preterm networks. The randomization was done by swapping the end-nodes of two randomly picked links, and keeping the weight with the link. In this way both the number of nodes, the number of links, and the degree distribution are conserved. To generating one random network, 20,000 link-swaps were performed, although a swap was only made if the transition was not present beforehand. Single full term and preterm networks were subtracted and we recorded the number of times the transition between 1-11 and 1-12 favored full term by the same amount or more, as what is seen in the real data. The p value is then given by the number of times the 1-11 to 1-12 transition was found to be equal or better in the random networks compared to the real network, and then divided by the total number of tests.

References