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An investigation on the regulation of the tweak–Fn14–NF-kB pathway

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AN INVESTIGATION ON THE REGULATION OF THE TWEAK–FN14–NF-κB PATHWAY

by

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Jee-Ching Wang

DEDICATION

To the memory of my grandfather, Shri Om Prakash Khetan. He was the first to suggest for me a career in science but departed us before the completion of my studies.

And to the ancient father, *Atharvan*, who instituted the earthly *Agni* and brought forth the practice of medicine with his words,

"I have brought you here; I have found you; you have come back renewed; you with healthy limbs! I have found your sight healthy and your lifetime whole."

– *Atharvaveda* VIII.I.XX

PUBLICATION DISSERTATION OPTION

This dissertation consists of the following two articles, formatted in the style used by the Missouri University of Science and Technology.

Paper I: Pages 7-34 have been published in the Journal of Theoretical Biology.

Paper II: Pages 35-58 are intended for publication soon.

ABSTRACT

Dysregulation of inflammatory pathways is strongly implicated in cancers and autoimmune diseases. The most consequential of these pathways involves the nuclear translocation of NF-κB, a transcription factor that induces the transcription of multiple proteins associated with cell survival, inflammation, proliferation and death. It is activated when the fibroblast growth factor-inducible 14 kDa protein (Fn14), a trimeric receptor recruits its ligand, TWEAK. Studies have shown that Fn14 is over-expressed in many tumors, the aggressiveness of which is often correlated with the degree of upregulation. Furthermore, TWEAK-Fn14 activation has been shown to result in persistent NF-κB activation. Using a mechanistic model of the signaling system, two specific features of the Fn14 pathway, (a) the ability of Fn14 constitutive signaling and (b) NF-κB induced de novo Fn14 expression were identified that give rise to positive feedback regulation and differentiate it from TNF-α receptor signaling. Further analysis revealed that stimulation of Fn14 by TWEAK may generate highly non-linear dynamics, including stable limit cycles and bistable responses. Another critical contributor to the Fn14 signaling dynamics was found to be TWEAK-dependent Fn14 trafficking dynamics. Rapid internalization allowed cells to show only transient NFκB activity while lack of internalization was a significant factor in maintaining the cell in a constitutively active state. Detailed study of Fn14 internalization, recycling and degradation allowed the creation of a more comprehensive signaling model which is capable of accounting for a wide range of Fn14 signaling behaviors observed in pathological tissues. The model was further used as a platform for *in silico* studies of the effects of potential targeted therapies on constitutively active cells such as those found in aggressive tumors. The model predicts that two conventional therapeutic approaches – Fn14 antagonists and anti-Fn14 siRNA – would provide unsatisfactory benefits while a novel approach involving targeted degradation of the receptor could be promising avenue for developing anti-Fn14 therapeutics.

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I must also acknowledge the invaluable assistance provided by Dr. Md. Shahinuzzaman and Dr. Aminul Islam in the intricacies of systems biology and BNGL programming. Also, my heartfelt thanks to Dr. Muhammad Raisul Abedin for the wealth of biological knowledge he shared with me throughout my studies. Many of our discussions have made their way into this dissertation, whether directly or indirectly.

Lastly, I would like to acknowledge the help and support of my parents who, despite being thousands of miles away, through their relentless encouragement were the constant motivating force behind my doctoral studies.

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1. INTRODUCTION

One of the hallmarks of all cancers is the ability of tumors to induce a persistent inflammatory environment [Hanahan and Weinberg, 2011]. Inflammation causes the immune system to supply neoplastic lesions with oncogenic bio-molecules such as survival factors that help evade apoptosis, growth factors that help sustain proliferation, pro-angiogenic factors which enhance blood supply to the tumor, and inductive signals that lead to activation of epithelial-mesenchymal transition [Grivennikov *et al.*, 2010]. A crucial mechanism employed by tumor cells to accomplish this is the persistent activation of the NF-κB pathway [Xia *et al.*, 2014]. The NF-κB transcription factor induces the expression of a number of pro-inflammatory cytokines which recruit the innate and adaptive immune-system to enable tumorigenesis. Among the large number of cytokine-receptor systems that can induce NF-κB activation is the receptor Fn14 and its ligand, TWEAK.

1.1. THE TWEAK CYTOKINE AND THE FN14 RECEPTOR

Tumor necrosis factor (TNF)-related weak inducer of apoptosis (TWEAK) is a pro-inflammatory cytokine excreted by various tissues especially in response to injury [Chicheportiche *et al.*, 1997]. It binds with the fibroblast growth factor-inducible 14 (Fn14) receptor (Figure 1.1) which is a type I transmembrane protein of the tumor necrosis factor receptor (TNFR) superfamily [Bossen *et al.*, 2006]. Although Fn14 is found at low levels throughout the body, it is particularly over-expressed in solid tumors and at the site of tissue injury [Perez *et al.*, 2016]. In particular, Fn14 overexpression is seen in lung, breast and oesophagal cancer, melanoma and glioblastoma [Tran *et al.*, 2003, 2006; Whitsett *et al.*, 2012; Zhou *et al.*, 2014]. In fact, Fn14 levels are highest in the most aggressive cancers such as metastasized breast, lung and colorectal cancers [Whitsett *et al.*, 2014; Yin *et al.*, 2014; Zhou *et al.*, 2013], and these levels correlate strongly with poor prognosis for the patient [Perez *et al.*, 2016; Pettersen *et al.*, 2013; Tran *et al.*, 2006; Watts *et al.*, 2007].

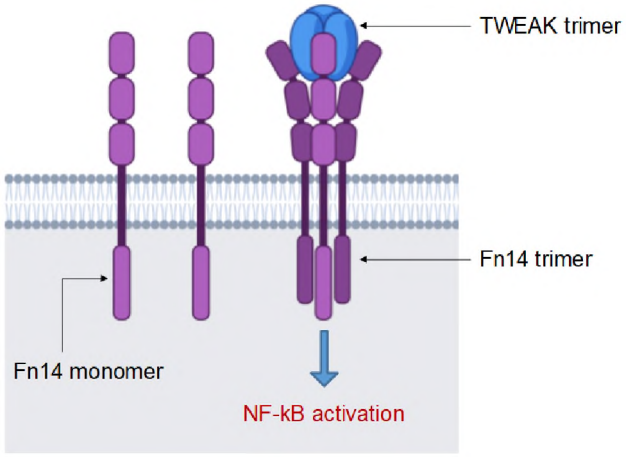

Figure 1.1. TWEAK-Fn14 interaction. The trimeric ligand TWEAK is recruited by Fn14 receptors on the cell surface, usually found as monomers. TWEAK then induces trimerization by recruiting nearby Fn14 monomers to the complex. The receptor trimer is the active signaling species.

Activation of Fn14 by TWEAK recruitment to the cell plasma membrane triggers multiple intracellular signaling pathways associated with cell growth, proliferation, migration, and apoptosis [Burkly *et al.*, 2007; Donohue *et al.*, 2003; Justo *et al.*, 2006; Polek *et al.*, 2003; Tran *et al.*, 2003, 2005]. This is analogous to other members of the TNF ligand superfamily such as TNF-α and its receptor TNF-αR which is the most extensively studied member or this family. However, TWEAK-Fn14 signaling differs in crucial ways to TNF-α–TNF-αR signaling. The most salient distinction between the two pathways is that stimulation of Fn14 by TWEAK induces persistent NF-κB activation [Colleran *et al.*, 2011; Dogra *et al.*, 2006; Gomez *et al.*, 2016; Saitoh *et al.*, 2003; Sanz *et al.*, 2008; Tran *et al.*, 2006]. This is in contrast to the more-well studied TNF-αR system where a number of studies show a transient NF-κB activation in response to sustained TNF-α stimulation [Lee *et al.*, 2000, 2014; Maecker *et al.*, 2005; Quivy *et al.*, 2002; Rogers and Fuseler, 2007; Saitoh *et al.*, 2003].

Despite the many similarities in the downstream protein interactions of the Fn14 and the TNFα pathways, there is experimental evidence for two uniqe interactions in the Fn14 pathway which could give rise to the significantly different behaviors. Firstly, Fn14 can activate without requiring stimulation by TWEAK when it is overexpressed on the cell surface [Brown *et al.*, 2013; Winkles, 2008]. Secondly, Fn14 induced nuclear localization of NF-κB can itself induce the expression of the Fn14 gene and de novo synthesis of the protein [Tran *et al.*, 2006]. Thus, it can potentially give rise to positive feedback regulation in the system [Kwon *et al.*, 2014; Tran *et al.*, 2006]. We may hypothesize that a cell when stimulated with TWEAK activates the NF-κB pathway resulting in Fn14 expression. This may result in Fn14 overcrowing on the plasma membrane which may allow the signal to be sustained without requiring TWEAK stimulation.

1.2. MODELING THE NF-κB PATHWAY

The localization of the transcription factor NF-κB to the nucleus is a central step in most immune responses [Pahl, 1999]. This can be triggered by a diverse range of stimuli such as viral [Hiscott *et al.*, 2006] or bacterial infections [Laflamme and Rivest, 2001], inflammatory cytokines, free radicals [Bubici *et al.*, 2006] and DNA damage [Bender *et al.*, 1998]. NF-κB is ubiquitously present in an inactive form in the cytoplasm bound to its inhibitor IκBα. Activation of the pathway leads to destruction of IκBα thereby allowing NF-κB to rapidly translocate to the nucleus where it binds to specific sites on the genome and triggers transcription of various genes [Brasier, 2006].

Since the first mathematical model of the NF-κB pathway was published by Hoffman et al in 2002 [Hoffmann *et al.*, 2002], significant advances have been made by incorporating more protein interactions in the model resulting in comprehensive models that can account for a wide range of experimental observations [Basak *et al.*, 2007; Cheong *et al.*, 2008; Hoffmann *et al.*, 2006; Kearns *et al.*, 2006; O'Dea *et al.*, 2007]. With the inclusion of the inhibitors IκBα and A20, these models show oscillations in NF-κB levels which was seen in

single cells in wet experiments [Hoffmann *et al.*, 2006] but not at the population level. This was resolved by Paszek et al [Paszek *et al.*, 2010] using a semi-stochastic method which accounts for cell to cell variation. They found that the heterogeneous oscillations at the single cell level can give the impression of non-oscillatory behavior at the cell population level [Williams *et al.*, 2014]. Most recently, Basak et al. [Basak *et al.*, 2007] and Shih et al. [Shih *et al.*, 2012] have augmented the model with the inclusion of the p100/IκBδ protein which degrades to induce nuclear localization of non-canonical NF-κB.

1.3. MOTIVATION TO STUDY FN14 SIGNALING

The above mentioned models however deal exclusively with the TNF-αR system, with the implication being that since other members of the TNF superfamily behave similarly, these models could act as satisfactory proxies for studying their signaling dynamics as well. However, with the discovery of certain unique aspects of TWEAK-Fn14 signaling such as ligand independent activation [Brown *et al.*, 2013; Winkles, 2008], Fn14 de novo synthesis [Tran *et al.*, 2006] as well as non-canonical pathway activation [Burkly, 2015], the Hoffman model may not be sufficient to explain the peculiar experimental behavior of TWEAK-Fn14. Given the implications of Fn14 signaling in very lethal pathologies, it has become necessary to develop a computational model which incorporates the most up-to-date evidence of Fn14 signaling behavior. This has been the primary motivation for the work presented in this dissertation.

1.4. RULE-BASED MODELING FOR SYSTEMS BIOLOGY

Rule-based modeling is a simple yet powerful approach to study dynamic biological systems. A typical approach involves specifying a network of rational reaction rules and species which follow these rules. This is known as the network topology. Sometimes, the rates of these governing reactions are known but often they are not. In the latter case, the numerical values of these rate constants are varied over a wide range and the general

system behavior is observed. Deliberately varying these numbers reveals regions of sytem behavior which might mimick experimental observations such as oscillations, switch on or off behavior, noise filtering etc.

In the present study, the model was defined in BNGL [Faeder *et al.*, 2009]. BNGL is a machine-readable language that can be executed using several rule-based modeling tools [Blinov *et al.*, 2004; Colvin *et al.*, 2010; Sneddon *et al.*, 2011]. The language permits the creation of mechanistic models considering the site-specific details of the molecules and network species [Faeder *et al.*, 2005]. In a BNGL-defined model, the protein molecules are specified with coarse-grained features, such as domains and motifs. Rules are specified to define the interaction and transformations of these features. We executed the model using the rule-based modeling software BioNetGen [Harris *et al.*, 2016]. BioNetGen evaluates BNGL rules and calculates all possible species and reactions that can generate from the site-specific interactions and transformations of the molecules in a model. The software then generates a system of ordinary differential equations which govern the concentrations of the species according to the specified reaction rules.

1.5. WHY BUILD COMPUTATIONAL MODELS?

With the advent of data-rich biological research techniques, such as DNA sequencing, RNA sequencing and proteomics, computational models of biological processes are becoming invaluable to scientific progress. In this data-rich landscape, the ability to recognize trends and patterns is an increasingly important means to generate hypotheses which can be tested empirically using traditional techniques. Biological models are by their nature incomplete, but so is human comprehension of the processes governing biological systems. Instead, the key intellectual objective of modeling is to develop a system of interacting essential components bound by rational rules which may account for the largest possible set of experimental observations. Such a model may also provide a framework for interpreting new biological data. Computational models may also allow the determination of the rel-

ative importance of various system components. Often, seemingly small perturbations in a single component can have an enigmatically large effect on the system. For example, a mutation offering even a minute survival advantage can rapidly take over a tumor [Waclaw *et al.*, 2015]. A simple set of interaction rules however is sufficient to explain the darwinian takeover of advantageous mutations. In this way, interventions to a system can be focused to target specific components which may give the most promising results. Conversely, models can also help 'weed-out' unpromising hypotheses. Multiple interventions to a system may be tested *in silico* and those which target the less significant components will likely produce the least effective results. In this way, computational models are powerful tools to inform potential experimental design. This is especially true for biological models of disease progression which can provide recommendations for potential therapeutic approaches.

PAPER

I. ANALYSIS OF FN14–NF-κB SIGNALING RESPONSE DYNAMICS USING A MECHANISTIC MODEL

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ABSTRACT

Fn14 is a transmembrane receptor protein belonging to the tumor necrosis factor receptor (TNFR) superfamily. Many experimental reports have shown that crosslinking of the receptor by its extracellular ligand TWEAK induces prolonged activation of transcription factor NF-κB. On the other hand it has been reported that the TNF-α receptor, which is a more well-characterized member of the TNFR family, only transiently activates NF-κB in response to TNF-α stimulation despite sharing many similar molecular interactions with Fn14. Here, we investigate molecular mechanisms that enable Fn14 to display such behavior. In particular, we focus on two specific features of the Fn14 pathway, which are absent in the TNFR system, that potentially give rise to a positive feedback regulation. By developing a mechanistic model, we analyze how these features may determine the dynamics of an Fn14–NF-κB response. Our analysis reveals that stimulation of Fn14 by TWEAK may generate highly non-linear dynamics, including stable limit cycles and bistable responses. The type of response depends both on the strength and duration of a TWEAK signal. Our predictions and analyses also show that the molecular interactions underlying the positive

feedback explain the prolonged activation of NF-κB under certain parameter regimes. In light of the model predictions, we propose possible deregulations of Fn14 leading to its overexpression in solid tumors and tissue injuries.

Keywords: Computational Modeling, Bifurcation analysis, Computational Modeling, Rule-based modelling, Glioblastoma, TNFRSF12A, Cell signaling, Systems Biology

1. INTRODUCTION

The fibroblast growth factor-inducible 14 (Fn14) is a type I transmembrane protein of the tumor necrosis factor receptor (TNFR) superfamily and a prominent marker for several pathological conditions, including glioblastoma multiforme [Burkly *et al.*, 2007; Feng *et al.*, 2000; Lu *et al.*, 2011; van Kuijk *et al.*, 2010; Winkles, 2008; Zheng and Burkly, 2008]. Activation of Fn14 in the cell plasma membrane triggers multiple intracellular signaling pathways associated with cell growth, proliferation, migration, and apoptosis [Burkly *et al.*, 2007; Donohue *et al.*, 2003; Justo *et al.*, 2006; Polek *et al.*, 2003; Tran *et al.*, 2003, 2005]. The receptor is activated by a soluble cytokine TWEAK, a member of the TNF ligand superfamily. Activated Fn14 then activates the canonical NF-κB pathway in a way similar to the TNF-α receptor (TNF-αR), which is an important and extensively studied receptor of the TNFR family.

Experimental studies spanning the past two decades highlighted a specific characteristic of the Fn14 signaling pathway. It has been demonstrated that stimulation of Fn14 by TWEAK generates prolonged signaling and NF-κB activation [Colleran *et al.*, 2011; Dogra *et al.*, 2006; Gomez *et al.*, 2016; Saitoh *et al.*, 2003; Sanz *et al.*, 2008; Tran *et al.*, 2006]. Recent experimental reports indicate that the canonical NF-κB signaling cascade is involved in this prolonged and sustained activation process [Colleran *et al.*, 2011; Dogra *et al.*, 2006; Gomez *et al.*, 2016; Maecker *et al.*, 2005; Sanz *et al.*, 2008; Tran *et al.*, 2006] although an earlier work indicates the non-canonical NF-κB pathway could generate such response [Saitoh *et al.*, 2003]. This reported behavior in Fn14-induced sustained NF-κB

activation differs from the more-well studied TNF-αR system in that in the latter case, a number of studies show a transient NF-κB activation in response to sustained TNF-α stimulation [Lee *et al.*, 2000, 2014; Maecker *et al.*, 2005; Quivy *et al.*, 2002; Rogers and Fuseler, 2007; Saitoh *et al.*, 2003]. Both Fn14 and TNF-αR share many common and similar protein-protein interactions that lead to NF-κB activation. Therefore, the specific underlying mechanisms in the TWEAK-Fn14 and TNFα-TNFαR systems that account for this difference in experimental observation remain to be understood. This modeling study was particularly motivated to investigate this unresolved question about Fn14 signaling.

Although activation of Fn14 and TNF-αR is followed by many common molecular interactions, experimental reports indicate two key features that are unique to Fn14 signaling. Unlike the TNF-αR, Fn14 can be activated in a ligand-independent manner when overexpressed in a cell [Brown *et al.*, 2013; Winkles, 2008]. Furthermore, activation of Fn14 can induce the expression of its own gene and de novo synthesis of the protein [Tran *et al.*, 2006]. Thus, it can potentially give rise to positive feedback regulation in the system [Kwon *et al.*, 2014; Tran *et al.*, 2006]. Although the key molecular interactions that give rise to these two distinct features are fairly well-established, their regulatory roles in Fn14 signaling remain unclear. We were particularly interested in dissecting these roles and underlying molecular interactions in determining the Fn14 signaling response dynamics. In particular, we sought to answer whether these features, unique to Fn14 only, could explain the postulated positive feedback regulation and the prolonged NF-κB activation mentioned earlier.

To date, a number of modeling studies analyzed the response behavior of the TNF-αR pathway [Lipniacki *et al.*, 2007; Pkalski *et al.*, 2013; Tay *et al.*, 2010]. By contrast, to the best of our knowledge, there has been no theoretical or computational modeling study on the Fn14 receptor system. In this work, we developed a computational model on the Fn14–canonical NF-κB signaling pathway. The model incorporates the detailed site-specific binding of TWEAK and Fn14 in the cell plasma membrane. It also incorporates

the signaling events associated with NF-κB activation, Fn14 transcription, and constitutive Fn14 assembly in the plasma membrane. We used the model to predict and analyze the system under normal conditions and several possible deregulations. Our analysis indicates that: (a) the above two features of the Fn14 pathway are tightly coupled and they together generate prolonged NF-κB activation as reported in [Colleran *et al.*, 2011; Dogra *et al.*, 2006; Gomez *et al.*, 2016; Saitoh *et al.*, 2003; Sanz *et al.*, 2008; Tran *et al.*, 2006] and (b) it predicts such behavior can occur even in response to a transient TWEAK signal. These two features jointly synergize a positive feedback loop that may generate highly nonlinear dynamics, including sustained oscillation (stable limit cycles) and bistable responses. Based on the model predictions and analysis, we provide possible explanations of prolonged NF-κB response in tissue injuries [Burkly, 2014; Hotta *et al.*, 2011; Jakubowski *et al.*, 2005; Mittal *et al.*, 2010a,b] and Fn14 overexpression in certain types of solid tumors [Michaelson and Burkly, 2009; Tran *et al.*, 2003].

2. METHODS

2.1. MODEL SPECIFICATION

We formulated the Fn14-NF-κB signaling pathway model as an ordinary differential equation (ODE) model. The model describes biochemical interactions and transformations of a list of signaling protein molecules in the Fn14-NF-κB signaling pathway. Each of these interactions and transformations is detailed in the Supplemental Material (Appendix A). An overall description of the model is provided in the Results section.

The model was defined in BNGL [Faeder *et al.*, 2009]. BNGL is a machine-readable language that can be executed using several rule-based modeling tools [Blinov *et al.*, 2004; Colvin *et al.*, 2010; Sneddon *et al.*, 2011]. The language permits the creation of mechanistic models considering the site-specific details of the molecules and network species [Faeder *et al.*, 2005]. In a BNGL-defined model, the protein molecules are specified

with coarse-grained features, such as domains and motifs. Rules are specified to define the interaction and transformations of these features. Supplemental Material (Appendix A) details BNGL notation of these molecules and the rules describing their non-covalent binding and biochemical transformations. We executed the model using the rule-based modeling software BioNetGen [Harris *et al.*, 2016]. BioNetGen evaluates BNGL rules and calculates all possible species and reactions that can generate from the site-specific interactions and transformations of the molecules in a model. The network of our model contains 45 distinct chemical species and 114 elementary reactions.

2.2. SIMULATION

Simulations were carried out using BioNetGen. The software generates ODEs describing the mass conservation of species in the network of a BNGL-defined model. It also performs simulation using the numerical ODE solver package CVODE [Cohen *et al.*, 1996].

3. RESULTS

3.1. THE NF-κB MODEL

As mentioned earlier, we were particularly interested in this pathway for its two distinctive features, which are not shared by the TNF-αR–NF-κB system. Figure 1 provides a simple illustration of these two features. Fn14 crosslinking and activation occur both in ligand (TWEAK)-dependent and independent manners. Moreover, Fn14 itself can induce its own gene expression via the NF-κB pathway. Figure 2 details the molecular interactions in the model showing how they give rise to these two features.

The model incorporates site-specific binding between TWEAK and Fn14 in the cell plasma membrane. Because TWEAK naturally exists as a trimeric complex in solution, we represent it by a single ligand molecule containing three identical Fn14 binding

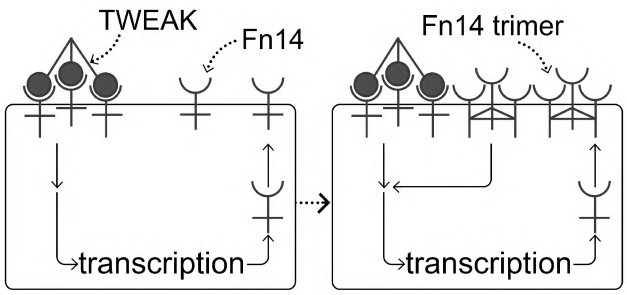

Figure 1. Receptor crosslinking and positive feedback in Fn14 signaling. Left: Ligand recruitment activates NF-κB pathway promoting receptor transcription. Right: Receptor overexpression leads to self-association and constitutive activity.

sites (Figure 2). These three sites sequentially bind three Fn14 molecules to form a 1:3 ligand-receptor complex. However, the interaction also generates intermediate 1:1 and 1:2 ligand-receptor complexes. Nevertheless, only the 1:3 complex represents the signaling-competent species [Winkles, 2008]. Besides this TWEAK-mediated crosslinking, we also consider constitutive Fn14 assembly that occurs in the absence of TWEAK (Figure 2). For each Fn14 molecule, we consider two identical binding surfaces via which it can assemble with other Fn14 molecules and generate receptor homodimers or homotrimers. The homotrimers undergo a ring-closure reaction (Figure 1) to generate a relatively more stable complex. As in the case of ligand-mediated receptor crosslinking, only the homotrimers represent signaling competent species. We consider all ligand-dependent and -independent interactions reversible. Table A.2 provides a list of the forward and reverse rate constants associated with these interactions. For the ligand-independent association, we consider an affinity (equilibrium dissociation constant K_D^*) such that only 2% of the Fn14 molecules remain in the trimeric form under the nominal condition. This nominal condition represents the basal inactive state of the system.

The molecular events downstream of Fn14 trimerization belong to the classical NF-κB pathway (Figure 2). We incorporate most of these events based on the TNF-αR–NF-κB model of Tay et al. [Tay *et al.*, 2010]. Briefly, Fn14 trimers activate a cytosolic protein called IκB kinase kinase (IKKK). The activated IKKK (denoted as IKKKa in

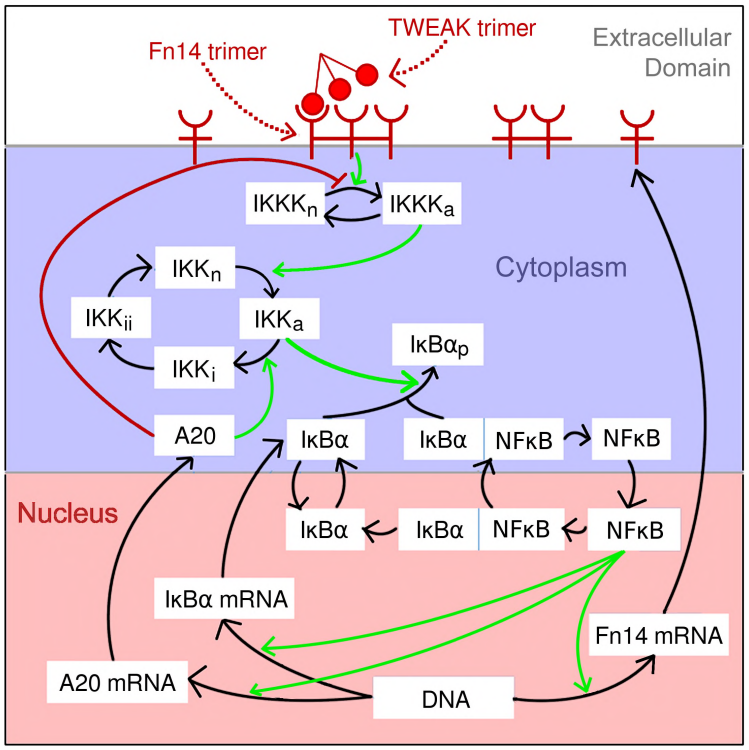

Figure 2. Signaling protein interactions incorporated in the Fn14-NF-κB pathway model. Besides the ligand and receptor, there are 9 distinct intracellular molecules whose functional states and interactions are illustrated. A black arrow indicates a transformation, a grey arrow indicates a positive influence and a blunt arrow indicates inhibition. The BNGL rules defining these interactions are provided in Appendix A, Computational Model.

Figure 2) directly phosphorylates and activates its downstream protein IKK. Subsequently, the activated IKK protein (denoted as IKKa) phosphorylates another protein IκBα, which is an inhibitor of NF-κB. In its basal unphosphorylated form, IκBα remains in complex with NF-κB and inhibits its nuclear translocation. However, phosphorylation of IκBα by IKK releases this protein from the complex, thus allowing NF-κB to translocate into the nucleus. After nuclear translocation, NF-κB induces transcription of A20, IκBα, and Fn14 (Figure 2). A20 serves as an inhibitor of IKKK. Therefore, its transcription activates a negative feedback in the system. Similarly, transcription of IκBα, which prohibits nuclear translocation of NF-κB, also activates a negative feedback. Finally, transcription of Fn14 activates a positive feedback, as explained before in Figure 1.

3.2. FN14 ACTIVATES SUSTAINED NF-κB RESPONSE AGAINST A TRANSIENT EXPOSURE TO TWEAK

One of our primary interests was to study how Fn14 self-assembly and the positive feedback in Fn14 gene expression may define the dynamical behavior of the system. These two features are expected to distinguish the system from the TNF-αR–NF-κB pathway. While a number of studies have shown that sustained TNF-α exposure may induce transient NF-κB activation [Lee *et al.*, 2000, 2014; Maecker *et al.*, 2005; Quivy *et al.*, 2002; Rogers and Fuseler, 2007; Saitoh *et al.*, 2003], it has been have reported that Fn14 stimulation by TWEAK yields prolonged NF-κB activation [Maecker *et al.*, 2005; Saitoh *et al.*, 2003]. We investigated whether the model could explain this experimental observation. We first predicted Fn14 and NF-κB responses under a pulse stimulation by TWEAK (Figure 3). Our simulation resembled a washout experiment, where cells were first stimulated with TWEAK for an hour and subsequently washed out by replacing the ligand-containing medium with a ligand-free fresh medium. The dotted curves in Figure 3A and B illustrate this pulse input.

Consistent with experimental reports, the model predicted prolonged Fn14 expression (Figure 3A) and NF-κB nuclear localization (Figure 3B). Despite the removal of ligand, the system did not return to its initial steady-state or basal condition. Instead, it reached a new state with significantly higher amounts of Fn14 and nuclear NF-κB. As mentioned previously, the basal condition in our model accounts for only 2% receptor trimerization via the constitutive receptor assembly. This small fraction of trimers maintains $\sim$ 0.2% of NF-κB in the nucleus in the absence of TWEAK. However, the model predicted an approximately 6-fold increase in Fn14 and a 14-fold increase in nuclear NF-κB after the stimulation (Figure 3A and B, respectively).

To further investigate the dynamics of the system, we systematically varied the dose and duration of the TWEAK pulse. The resulting responses revealed four distinct dynamical states for the system depending on the dose and duration (Figure 4). In response to a relatively weak or short pulse of TWEAK (10^{-3} ng/ml TWEAK for 1 min or 1 ng/ml

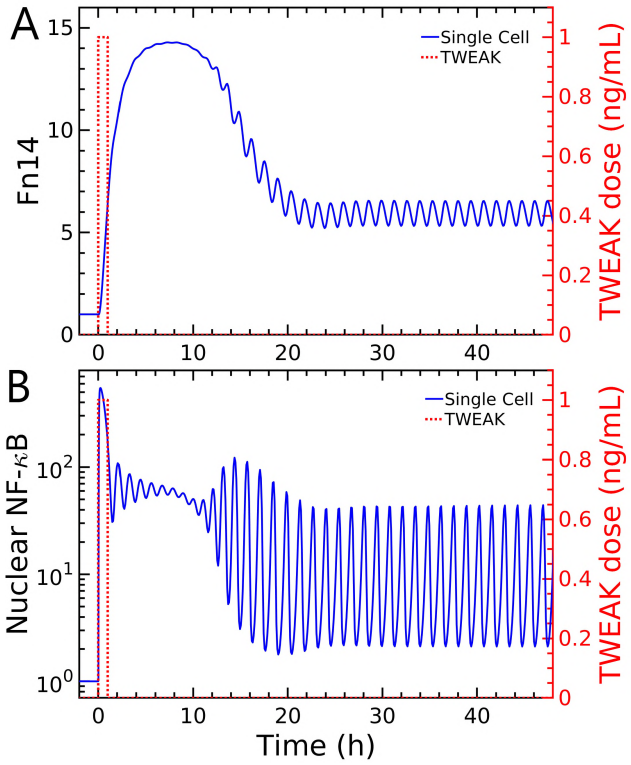

Figure 3. Predicted Fn14 and NF-κB dynamics in response to a pulse stimulation. In the simulation, cells were subject to a fixed concentration of extracellular TWEAK for 1 hour. The dotted grey curve in each panel represents this pulse input. The figure shows two different responses: (A) Fn14 expression and (B) NF-κB nuclear localization. Both responses are normalized by corresponding basal amounts.

TWEAK for 1 s), the system remained almost non-responsive. A moderate pulse (0.1 ng/ml for 1 min or 1 ng/ml for 5 s) activated a transient response after which the system returned to the initial basal state. A relatively strong pulse (1 ng/ml for 1-10 min) generated a sustained oscillation (stable limit cycles). Finally, a persistent stimulation (a step input of 1 ng/ml TWEAK) drove the system into a fully-activated steady-state condition.

Against a transient pulse of TWEAK signal, one would naturally expect to see a transient response. In contrast, our model predicts sustained responses against very short pulses of TWEAK (Figure 4).

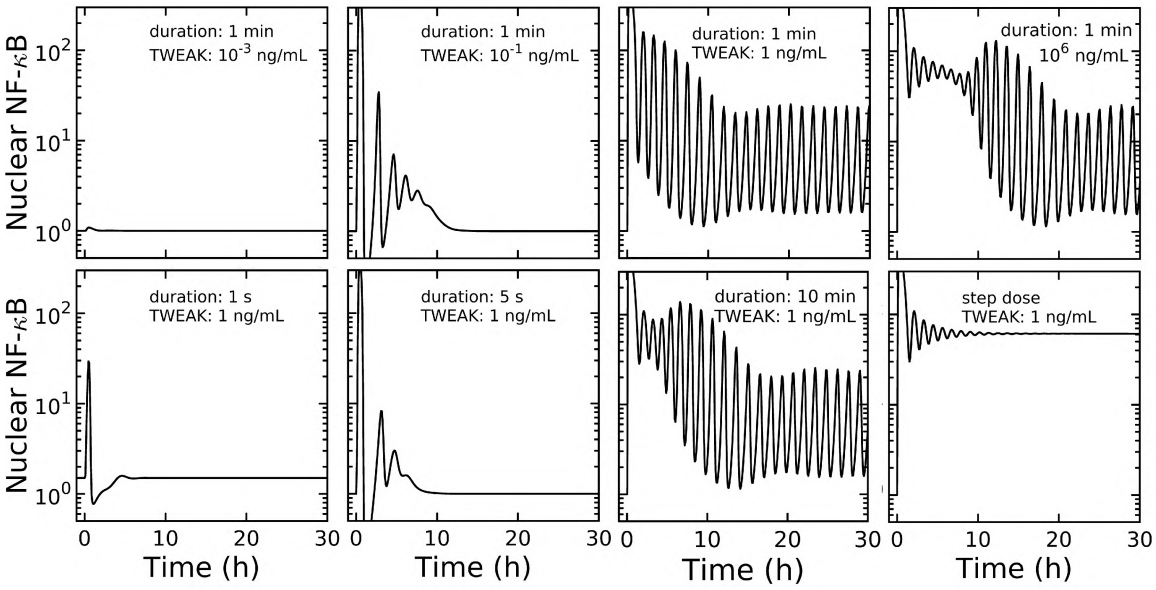

Figure 4. NF-κB dynamics under TWEAK pulses of different durations and strengths. The upper panels represent NF-κB dynamics under a short (1 minute) pulse of variable strengths. The lower panels represent NF-κB dynamics under a pulse of variable duration but fixed strength (1 ng/mL TWEAK).

3.3. CONSTITUTIVE ASSEMBLY OF FN14 AND ITS SELF-INDUCED GENE EXPRESSION ARE ESSENTIAL FOR THE SUSTAINED RESPONSE

Our predictions in Figure 4 provide an important insight into the molecular mechanism that could be responsible for the prolonged NF-κB activation. It is possible that the constitutive receptor assembly is the key to this behavior. Such assembly may prohibit the system from returning to the basal state after a transient stimulation. When a threshold number of Fn14 populates in the plasma membrane in response to a stimulation, the constitutive Fn14 assembly may generate an appreciable amount of receptor trimers. The receptor trimers may then induce the expression of Fn14 gene further, thus leading to a positive feedback loop in the system. As a result, the two features may perpetually sustain signaling even though the ligand is washed away or depleted from the extracellular space.

To investigate the speculations above, we used the model to predict how interventions in the two features above could impact the behavior of the system. Blocking Fn14 transcription by nuclear NF-κB should eliminate the positive feedback loop. On the hand, prohibiting constitutive Fn14 self-assembly should impair its potential role in prolonging responses discussed in the previous section. In the model, we implement the former intervention by setting the rate constant associated with Fn14 mRNA translation to zero. We implement the latter intervention by setting the forward rate constant for constitutive Fn14 assembly to zero.

Figure 5 compares the responses of the compromised cells created through the above two interventions. Both wild-type and compromised cells activated rapid responses immediately after TWEAK exposure. However, unlike the wildtype cell, none of the two compromised cells was able to sustain signaling. The difference became apparent at about $t > 10$ h. The response of the wildtype cell eventually settled down to a constant-amplitude oscillation (stable limit cycles) (Figure 5A). In contrast, the responses of the compromised cells returned back to the basal inactive state within 10 h of stimulation (Figure 5B).

The result indicates that either intervention was adequate to abolish a cell's ability to generate a self-sustained response. In the model, blocking Fn14 transcription by NF-κB essentially keeps the amount of Fn14 in a cell fixed regardless of TWEAK stimulation. Because the positive feedback is not activated, the system returns to its basal state after generating a transient signal. On the other hand, when the constitutive receptor assembly is blocked, the induced Fn14 expression in response to TWEAK does not contribute to signaling after the ligand is washed away and the system returns to its basal state as well. From Figure 5B, this latter intervention appears to have a more pronounced effect on signaling.

The oscillations in Figure 5 could arise from a negative feedback in the NF-κB cascade. This negative feedback is associated with transcription of A20, which acts as a negative regulator of IKKK (Figure 2). The oscillations are relatively fast compared to the

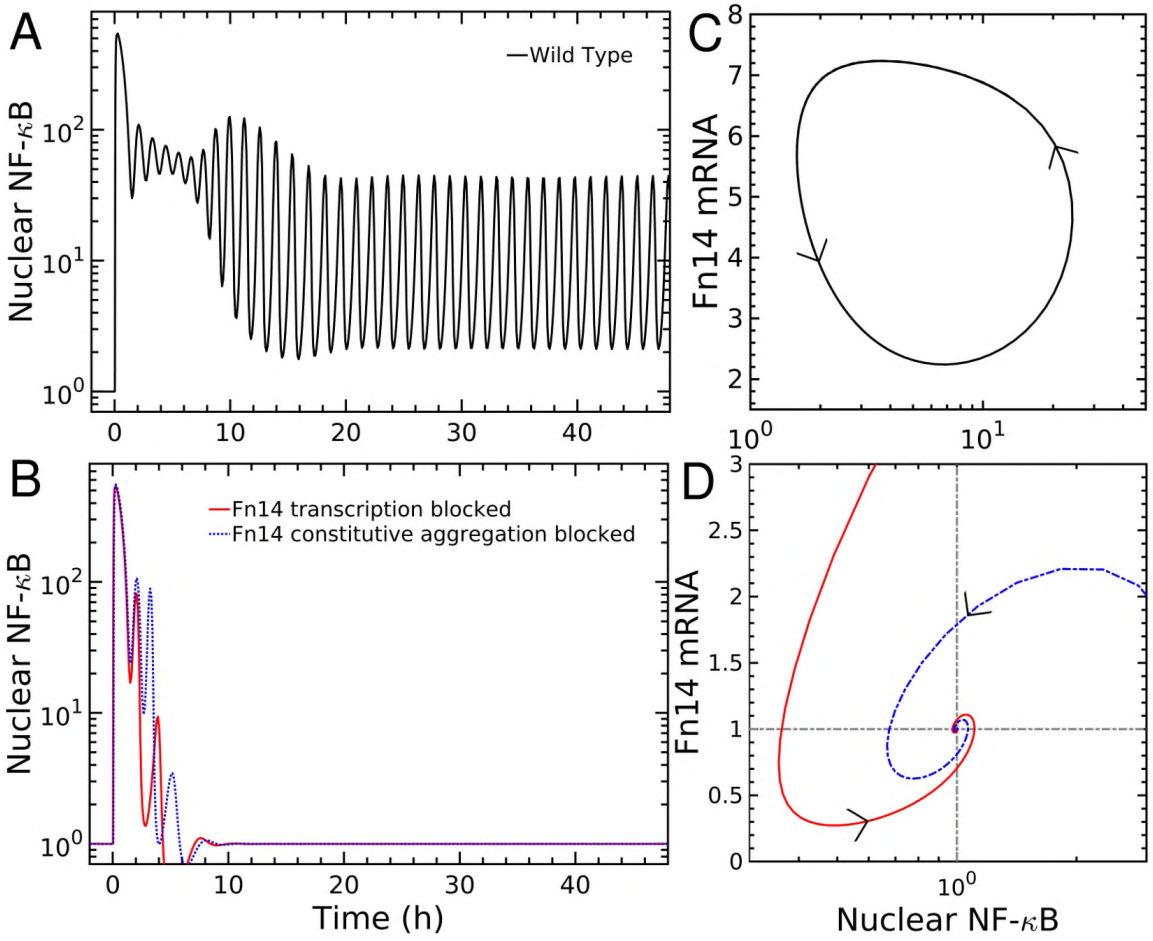

Figure 5. Comparison between wildtype and compromised (mutant) cell responses. All simulations represent stimulation by a 10 minute 1 ng/ml TWEAK pulse. (A) Post-stimulation nuclear NF-κB in a wildtype cell. (B) Post-stimulation nuclear NF-κB in compromised cells. The solid curve represents a cell where NF-κB-mediated transcription of Fn14 was blocked. The dotted curve represents a cell where Fn14 constitutive assembly was prohibited. (C) Phase portrait of Fn14 mRNA vs. nuclear NF-κB for a wildtype cell. (D) Phase portrait of Fn14 mRNA vs. nuclear NF-κB for the two compromised cells (the solid and dotted lines correspond to the cells in Panel B). In the panels, concentrations are presented in terms of relative values with respect to the basal state of corresponding cells.

slow dynamics of Fn14 expression and NF-κB nuclear localization. In the wildtype cell, the oscillations gradually turn into stable limit cycles with mean response significantly above the basal condition. On the other hand, in the compromised cells, the oscillations decay gradually and the system completely returns to the basal condition.

3.4. BIFURCATION ANALYSIS REVEALS BISTABILITY IN FN14-NF-κB SIGNALING

Biochemical network systems containing positive feedback regulations are often characterized by bistable responses. The rapid switch-like activation upon stimulation (Figure 5) motivated us to interrogate such a possibility in the system. We first considered K_D^* as a bifurcation parameter in our analysis. This parameter, which is the equilibrium dissociation constant for constitutive Fn14 self-assembly, defines the stability of Fn14 trimers formed in the absence of TWEAK. The value of K_D^* has not been quantified or reported in the literature. However, as mentioned earlier, we assigned it a nominal value such that a small fraction of receptors ($< 2\%$) are incorporated in trimers in the absence of TWEAK.

Figure 6A and B show bifurcation analysis on the wildtype system in the absence of TWEAK. The system displayed only stable limit cycles at $K_D^* < 4$ μM. On the other hand, it remained non-responsive and maintained the basal steady-state at $K_D^* > 7.5$ μM. In between these values of K_D^*, it displayed a coexistence of oscillation and nearly basal steady-state condition. It should be noted that the nominal value for K_D^* used in the model is 6.1 μM, which falls within the intermediate range.

We then investigated a second bifurcation parameter $\delta = r_1/r_2$, where r_1 and r_2 represent the rate of IKKK activation by the constitutively-formed and TWEAK-crosslinked Fn14 trimers, respectively (Table A.2, Appendix A). This parameter allows us to hypothetically consider a scenario where the signaling strength of a constitutively-formed Fn14 trimer is distinct from that of a TWEAK-crosslinked Fn14 trimer. Figure 6C and D show bifurcation plots for cellular Fn14 and nuclear NF-κB against δ. The plots indicate two distinct steady-state conditions in addition to the intermediate oscillatory regime. At $0 < \delta < 3 \times 10^{-4}$, the system remains in the non-responsive steady state. In contrast, at $\delta > 1.6 \times 10^{-3}$, the system displays a new steady-state condition, which corresponds to its fully activated state. The coexistence of a steady state and stable limit cycles is seen in the

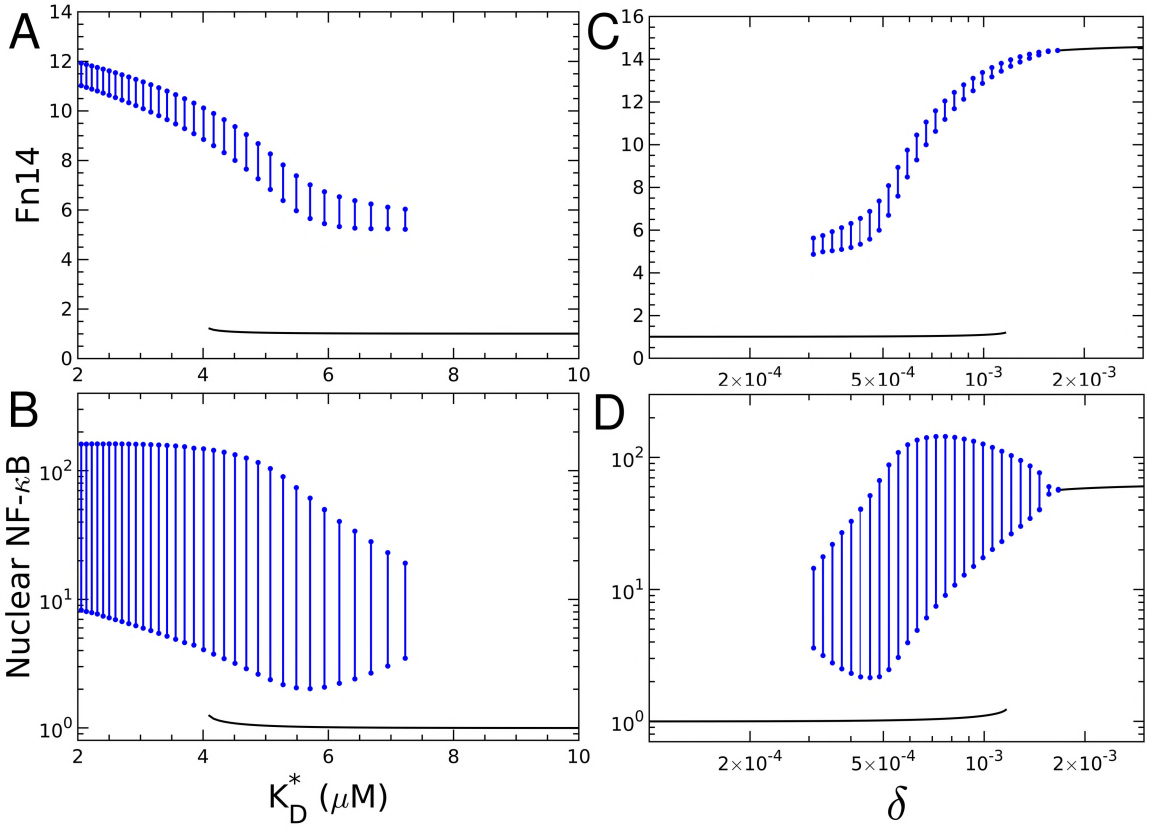

Figure 6. Bifurcation analysis. Fn14 level and nuclear NF-κB are analyzed against two bifurcation parameters K_D^* and δ. Parameter K_D^* represents the equilibrium dissociation constant (binding affinity) for constitutive assembly of Fn14. Parameter δ measures the signaling strength of a constitutively formed Fn14 trimer relative to a TWEAK-crosslinked Fn14 trimer. The four panels represent the following bifurcation curves: (A) Fn14 vs. K_D^*. (B) nuclear NF-κB vs. K_D^*. (C) Fn14 vs. δ. (D) nuclear NF-κB vs δ.

intermediate range $3 \times 10^{-4} - 1.2 \times 10^{-3}$. A purely oscillatory response is seen between $\delta = 1.2 \times 10^{-3} - 1.6 \times 10^{-3}$. In summary, these bifurcation analyses indicate three distinct long-time behaviors of the system. It can return to the non-responsive basal state, display sustained oscillation, or reach a fully activated steady-state condition.

3.5. MAPPING OF FN14-NF-κB SIGNALING DYNAMICS TO DISTINCT PARAMETER REGIMES

The three qualitatively distinct states revealed by our bifurcation analysis strongly depended on δ and K_D^*. Because the values of these two parameters are unknown, we wanted to characterize the regimes where the system might display the above three states. The mapping in Figure 7A shows how various combinations of these two parameters jointly determine these long-time behaviors for the wildtype system in the absence of TWEAK. Because the system should remain non-responsive without stimulation, these two parameters should not fall in the β and α regimes, where it generates stable limit cycles or fully activated steady-state response, respectively. However, it is possible that deregulation could change either of these parameters and drive the system into these regions even in the absence of stimulation. For example, a point mutation might lead to an increase in the affinity of receptor self-assembly, thus leading to a smaller K_D^*. As shown in the figure, at $K_D^* < 1.2$ μM, the system could become auto-activated regardless of the value of δ. However, the above mappings change dramatically in the presence of TWEAK, as shown in Figure 7B and C. Both these panels correspond to a 1-hour pulse of TWEAK but the concentrations are 1 ng/ml and 1 mg/ml, respectively. Considering the system becomes activated under such stimulations, it is unlikely that the two parameters fall in the non-responsive (γ) region of these two panels. Therefore, part of the γ region in Figure 7A that falls in the β or α region of the latter two panels could represent the feasible regime for these two parameters. In summary, these bi-parametric mappings indicate the sensitivity of the model to the choice of different combinations of two parameters K_D^* and δ.

However, to investigate the sensitivity of the system further to the choice of our model parameter values, we also investigated its responses against joint variations of the three parameters introduced in this study ($k_D^* = k_{-c}/k_c$, δ and d_f). Rather than systematically varying these three parameters, we assigned distribution for each of them and randomly sampled their values from these distributions. Figure 8A shows the assigned distributions

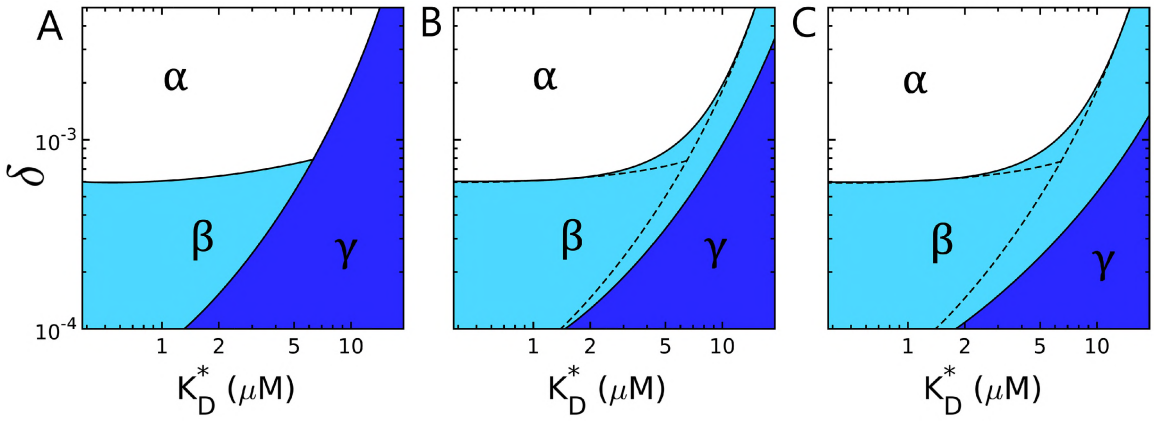

Figure 7. Two parameter mapping of long-time NF-κB dynamics. (A) Distinct types of NF-κB responses at $t \sim \infty$ under various combinations of parameters δ and K_D^* in the absence of TWEAK. The regions indicated by γ, β and α represent the basal steady-state, sustained oscillation (stable limit cycles), and activated steady state, respectively. (B) The same as Panel A when the system is stimulated for 1 hour with 1 ng/ml TWEAK. The dashed lines represent corresponding boundaries under zero TWEAK (Panel A). (C) The same as Panel B when the system is stimulated for 1 hour with 10^3 ng/ml TWEAK.

for these parameters. Each parameter was distributed log-normally with the mean taken as the base parameter value listed in the Supplemental Material (Appendix A). The log standard deviation was taken to be 0.3 for each parameter distribution. This standard deviation was chosen so that the parameters take values that differ by 2 to 3 orders of corresponding base values. Using the sampled parameters, we performed simulations considering a 10-minute transient TWEAK exposure. We sampled 1,000 distinct combinations of the three parameter values. Each sample essentially represents a distinct cell in a population whose parameter values take the above-specified distributions. Therefore, our simulations generated 1,000 distinct time-series responses (trajectories) for 1,000 distinct cells.

The results from our simulations are plotted in Figure 8. For the entire population of 1,000 cells above, we identified three possible responses - auto-activation independent of TWEAK, TWEAK-induced sustained activation, and TWEAK-induced transient activation (Figure 8B and C). Despite the wide distribution of the parameter values, the cells displayed any of these three distinct response behaviors. Therefore, regardless of the parameter value

we choose, the system is expected to generate any of these three qualitative behaviors. Figure 8C show that for each qualitative behaviors, the three parameters are clustered in the same region in the three-dimensional parameter space. These clusters are indicated by the three color codes (red, green and blue). In general, low values of K_D^* and d_f and high values of δ (the red cluster in Figure 8C) create a system that is always activated regardless of the presence or absence of TWEAK due to Fn14 overexpression. At intermediate values of these parameters (the blue cluster), the system is not activated in the absence of TWEAK but exhibits sustained activation in response to a transient TWEAK stimulation. At large values of K_D^* and d_f and low values of δ (green region), the system exhibits a transient response to a transient TWEAK stimulation. Panels D and E show corresponding results result in response to a step dose of TWEAK. As one might expect, cells do not return to the basal inactive state as long as the stimulation sustains.

4. DISCUSSION

In summary, we have provided a detailed analysis of how two unique regulatory features of the Fn14–NF-κB system define its dynamical responses. Our results indicate that Fn14's ability to induce its own gene transcription [Kwon *et al.*, 2014; Tran *et al.*, 2006] coupled with its ligand-independent self-assembly in the plasma membrane may give rise to a positive feedback loop in the system. This positive feedback self-sustains signaling after initial transient stimulation. Furthermore, positive feedback gives rise to highly nonlinear dynamics, including stable limit cycles and bistable responses in the system. The experimental work of Tran et al. [Tran *et al.*, 2006] first proposed that Fn14's ability to induce its own gene could give rise to a positive feedback regulation in the system. Here, using the model, we show that the effectiveness of this feedback may largely depend on the second feature, i.e., ligand-independent assembly of Fn14. The model indicates that the two features function in a complementary fashion to regulate NF-κB dynamics and Fn14 expression. Our analysis suggests that compromising either of the two features abolishes

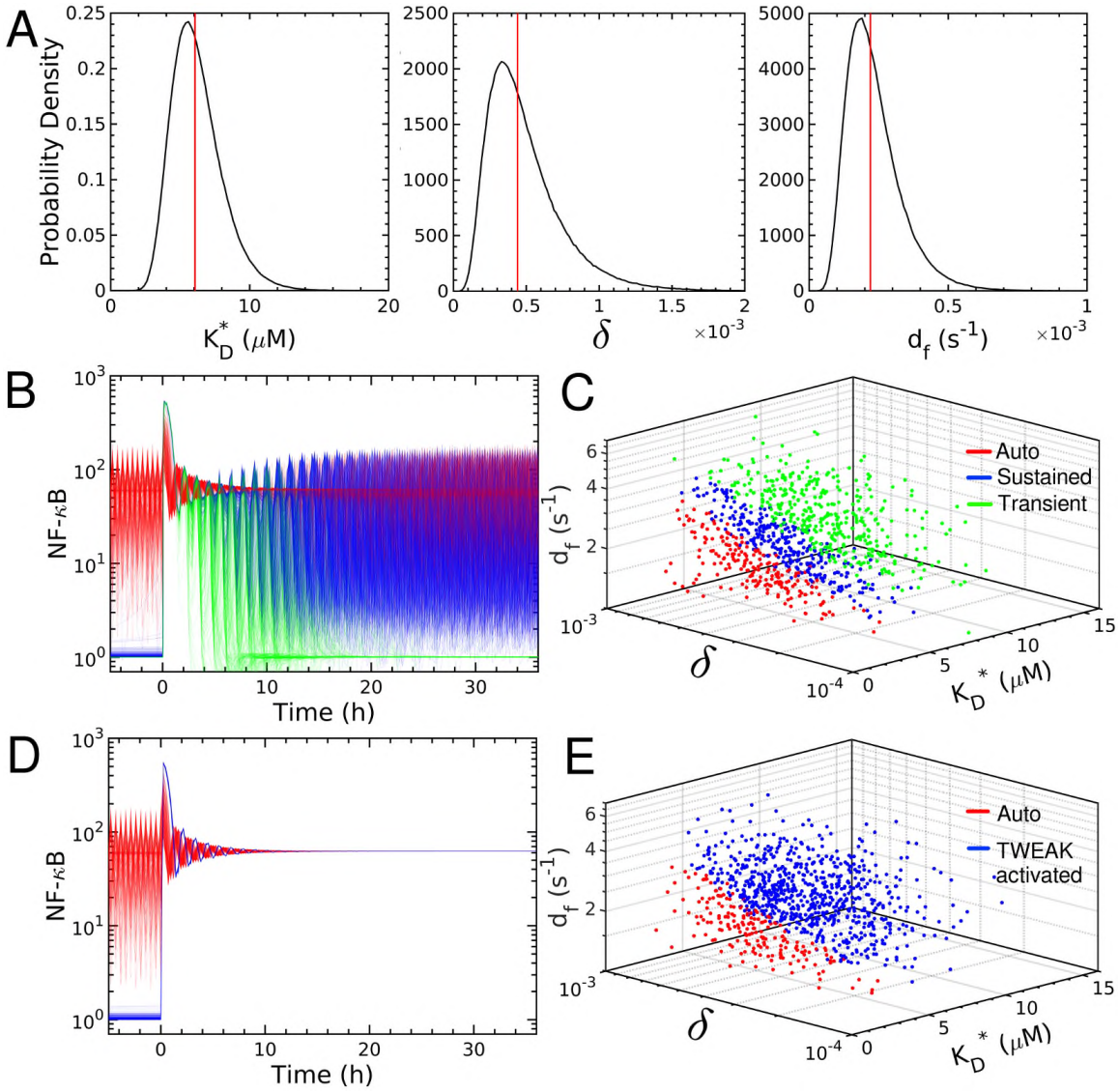

Figure 8. Sensitivity of the model predictions to its parameter values. The model was simulated using sampled values of K_D^*, δ, and d_f. A total of 1,000 combinations of these three parameters were sampled. Each sample represents a distinct cell in a population. (A) The assigned lognormal distributions of K_D^*, δ, and d_f. Vertical red bars represent the mean (nominal values in Supplemental Material (Appendix A). Each distribution corresponds to a log-scale standard deviation of 0.3. (B) Predicted responses considering 10-minute TWEAK stimulation. Colors represent three distinct qualitative behaviors revealed by the 1,000 simulated cells: ligand independent auto-activation (red), ligand-induced prolonged activation (blue) and ligand-induced transient activation (green). (C) Three dimensional scatter plot of showing the three distinct behaviors of the 1,000 simulated cells. The coordinate of each dot represents a distinct cell.

the positive feedback loop and the self-sustained response. In addition, mutations, which could potentially increase the affinity of Fn14 self-assembly, may lead to overexpression of Fn14 and trigger a self-sustained response even in the absence of TWEAK stimulation.

A key contribution of our work is that it mechanistically explains the TWEAK-induced prolonged activation of NF-κB, an observation reported in many experimental works [Colleran *et al.*, 2011; Dogra *et al.*, 2006; Gomez *et al.*, 2016; Saitoh *et al.*, 2003; Sanz *et al.*, 2008; Tran *et al.*, 2006]. Most of these studies have indicated the canonical NF-κB pathway involving in process. However, an earlier study by Saitoh et al. [Saitoh *et al.*, 2003] provides suggests a distinct mechanism involving the non-canonical pathway. Our model includes the elements of the canonical pathway only and hence our proposed mechanism aligns with these majority and more recent experimental studies [Colleran *et al.*, 2011; Dogra *et al.*, 2006; Gomez *et al.*, 2016; Sanz *et al.*, 2008; Tran *et al.*, 2006].

Importantly, the prediction for sustained response is valid for a definite parameter regime, as shown in Figures 7 and 8, where we have explored a wide range of our key model parameter values. It should be noted that our model is devoid of any element of the non-canonical NF-κB pathway. It would be interesting to extend our model to include this alternative pathway of NF-κB activation and study the mechanism reported in Saitoh et al. [Saitoh *et al.*, 2003]. However, the non-canonical NF-κB pathway is relatively less well-characterized in the context of Fn14 signaling. Therefore, we limited our model to the more well-established biological information associated with the Fn14-NF-κB signaling.

Our model indicates that positive feedback-induced bistability (Figure 6), essentially allows the system to respond in a switch-like manner (Figure 4). Depending on the stimulation dose or duration, the model predicts four distinct response behaviors for the system (Figure 4: 1) a basal or inactive steady-state; 2) a single pulse response, 3) sustained oscillation or stable limit cycles, and 4) fully-activated steady-state condition. Below a threshold level of exposure time, TWEAK is unable to activate the system. A weak and short TWEAK pulse above this threshold may generate a single pulse response so that

the returns to the basal inactive condition. A moderately strong stimulation or exposure time may give rise to sustained oscillation or stable limit cycles. Finally, a relatively long stimulation or step change in TWEAK drives the system into a fully-activated steady-state regime. Both the stable limit cycles and the fully-activated regime may self-sustain even if TWEAK is depleted or washed away after the temporal stimulation. This differs from the TNF-α autocrine positive feedback loop [Coward *et al.*, 2002; Rushworth *et al.*, 2011; Wu *et al.*, 1993] which is entirely ligand-mediated and therefore is dependent on extracellular factors such as extracellular ligand concentration, proximity of other active cells etc. Our TWEAK-Fn14 model predicts that activation could be sustained even after complete washout of ligand which is generally not the case with transient TNF-α stimulation [Chatterjee *et al.*, 2019; Hoffmann *et al.*, 2002; Poppers *et al.*, 2000; Turner *et al.*, 2010; Werner *et al.*, 2008].

The distinct types of responses predicted by the model underscore a potential filtering mechanism for cells. In normal physiological tissue, the positive feedback may distinguish signaling cues from noise [Hornung and Barkai, 2008]. It may keep a cell non-responsive under the stochastic noise of extracellular TWEAK that is not intended to activate signaling. However, in a tissue injury, it may recognize a stronger or longer TWEAK signal and trigger long-lasting responses. As shown in Figure 5, a few minutes of TWEAK exposure can lead to self-sustained NF-κB activation and Fn14 expression (Figure 5). These results highlight potential regulatory mechanisms whereby cellular expression of Fn14 could be controlled in a normal condition or tissue injuries. In a tissue injury, a prolonged NF-κB activation and Fn14 overexpression could be necessary for the healing process.

It should be noted that in most experimental settings, cells are exposed to a solution containing a fixed amount of TWEAK for a certain period of time. The duration of such exposure is typically longer than a minute. Our model indicates that stimulation for a small duration could lead to self-sustained NF-κB activation. We propose that such experiments should be done under short pulses of TWEAK stimulation. A systematic variation in

the pulse duration and its impact on specific readouts, such as NF-κB activation or Fn14 expression, could provide new insights and validate the predicted molecular mechanisms generating prolonged responses.

To date, it remains unclear what causes Fn14 overexpression in certain types of solid tumors. It is possible that a tumor is subject to high fluxes of TWEAK signals. However, besides TWEAK exposure, there could be other mechanisms behind such deregulation. Our model indicates that the affinity of constitutive Fn14 assembly is critical for the positive feedback loop (Figure 7). An increase in the affinity could make the system sensitive to the low level of TWEAK. One can surmise a naturally occurring point mutation in Fn14 altering the affinity. Such a mutation may induce constitutive Fn14 trimerization beyond a threshold and activate the system in the absence of TWEAK (Figure 7). An interesting study will be to investigate if such mutations indeed occur in glioblastoma and other solid tumor tissues, where Fn14 is routinely found overexpressed.

We propose two interventions in the pathway to investigate the mechanisms determining its dynamics. Our predictions in Figure 6 and Figure 7 indicate how the system response could be controlled by modulating the parameters δ and K_D^*. Between these two, K_D^* represents a more easily manipulable parameter. In the simulations, by altering the value of K_D^*, we modulated the effectiveness of the positive feedback loop. An experimental analog will be to introduce a mutation in the Fn14 molecule to alter K_D^* and compromise its ligand-independent assembly. Signaling responses resulting from such mutations may provide valuable insights into Fn14 deregulation and overexpression in solid tumors and other pathological conditions. Another intervention we propose is to block NF-κB-mediated expression of the Fn14 gene. This should also abolish the positive feedback and the possibility of auto-activation in the absence of TWEAK.

Our model, to our knowledge, is the first model developed for this system and it may serve as an initial base-case model at this point. The model can be refined and extended as new information becomes available. The ligand-receptor interactions of this model can be

adapted to create models for other pathways that operate downstream of Fn14. Examples include Fn14-mediated regulation of the non-classical NF-κB signaling pathway [Roos *et al.*, 2010] and the mitogen-activated protein kinase (MAPK) cascade.

Here, we formulated the model using the rule-based approach. The primary motivation was to incorporate the site-specific TWEAK–Fn14 interaction and assembly in the plasma membrane. However, we treated the rest of the protein molecules as featureless chemical species following an earlier model of TNF-α receptor–NF-κB signaling [Tay *et al.*, 2010]. An extension considering the site-specific details of these downstream protein molecules will provide a more predictive power and enable interrogation of the effects of targeted mutations at binding domains or motifs of the molecules [Barua *et al.*, 2007; Barua and Hlavacek, 2013]. Nevertheless, such comprehensive models are computationally challenging due to the large state-space dimension, as highlighted in [Faeder *et al.*, 2005]. Recently, we and others have developed a model reduction technique to avoid such complexity in rule-based models [Erickson *et al.*, 2019]. Alternatively, a tool implementing network-free stochastic simulation, such as NFsim, could also be employed to incorporate such details in the model [Sneddon *et al.*, 2011].

5. CONCLUSION

This modeling study unravels a possible molecular mechanism that enables the Fn14 receptor to generate prolonged NF-κB responses after a transient stimulation. Although many experimental works reported this behavior, the underlying molecular mechanisms remained poorly understood. The study finds that Fn14's ability to self-assemble in the absence of its ligand TWEAK is a critical feature in determining the sustained NF-κB response. The model predicts that Fn14 self-assembly along with de novo Fn14 synthesis in response to Fn14 stimulation synergistically activates a positive feedback loop, which contributes to the long-lasting signaling. The analyses reveal highly non-linear dynamics, including stable limit cycles and bistable responses that could be activated by the positive

feedback loop depending on the strength and duration of the extracellular TWEAK signal. The study sheds lights into the mechanisms whereby de novo Fn14 synthesis can lead to prolonged overexpression of the receptor in solid tumors and tissue injuries.

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AUTHOR CONTRIBUTIONS

J.K. carried out modeling and data analysis. D.B. conceptualized and supervised the work. All authors contributed to manuscript preparation and revision.

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II. DEVELOPMENT OF A COMPREHENSIVE TWEAK–FN14–NF-κB SIGNALING MODEL

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ABSTRACT

TWEAK is a pro-inflammatory cytokine belonging to the TNF-superfamily and is known to induce apoptosis, survival, proliferation and migration. It does so by interacting with its only known receptor, Fn14. TWEAK-Fn14 interaction is known to induce activation of the NF-κB pathway which results in the transcription of various pro-inflammatory proteins. Studies have shown that TWEAK-Fn14 interaction promotes multiple hallmarks of cancer such as invasion and migration, epithelial-mesenchymal transition and angiogenesis. Unlike the better studied TNFα-NF-κB pathway, Fn14 activation often results in persistent NF-κB activation, This may be due to unique features like constitutive signaling and self-induced Fn14 up-regulation, but no comprehensive computational models have been published which might illuminate these mechanisms. Here we propose a detailed TWEAK-Fn14-NF-κB model which takes into account its unique signaling behavior, rapid receptor trafficking as well as the relative contributions of the canonical and non-canonical NF-κB activation. We find that TWEAK does not cause significant Fn14 degradation which allows prolonged NF-κB activation via the canonical pathway. Furthermore, single-cell data reveals that the non-canonical NF-κB is only a small contributor to overall nuclear NF-κB levels. Our computational model faithfully describes the different behaviors of Fn14-inactive and constitutively active cells found in tumors. We further perform *in silico*

tests of three targeted pathway attenuating therapies. We find that conventional therapies such as receptor antagonists and siRNA treatment give modest results while a novel treatment involving targeted lysosomal degradation provides rapid and long-lasting pathway attenuation.

Keywords: Computational Modeling, Rule-based modelling, Glioblastoma, TNFRSF12A, Cell signaling, Systems Biology

1. INTRODUCTION

TWEAK is a pro-inflammatory cytokine excreted by various tissues especially in response to injury [Chicheportiche *et al.*, 1997]. It activates the inflammatory NF-κB pathway by binding to its only known receptor, Fn14[Bossen *et al.*, 2006]. Fn14 is known to be significantly overexpressed in lung, cervical and oesophagal cancer, melanoma and glioblastoma [Tran *et al.*, 2003, 2006; Whitsett *et al.*, 2012; Zhou *et al.*, 2014]. High levels of Fn14 are correlated with cancer aggressiveness such as in metastasized cervical, lung and colorectal cancers [Whitsett *et al.*, 2014; Yin *et al.*, 2014; Zhou *et al.*, 2013] as well as with poor prognosis for the patient [Perez *et al.*, 2016; Pettersen *et al.*, 2013; Tran *et al.*, 2006; Watts *et al.*, 2007]. Activation of Fn14 by TWEAK recruitment to the cell plasma membrane triggers multiple intracellular signaling pathways associated with cell growth, proliferation, migration, and apoptosis [Burkly *et al.*, 2007; Donohue *et al.*, 2003; Justo *et al.*, 2006; Polek *et al.*, 2003; Tran *et al.*, 2003, 2005]. This is analogous to other members of the TNF ligand superfamily such as TNF-α and its receptor TNF-αR which is the most extensively studied member or this family. However, TWEAK-Fn14 signaling differs in crucial ways to TNF-α–TNF-αR signaling. The most salient distinction between the two pathways is that stimulation of Fn14 by TWEAK induces persistent NF-κB activation [Colleran *et al.*, 2011; Dogra *et al.*, 2006; Gomez *et al.*, 2016; Saitoh *et al.*, 2003; Sanz *et al.*, 2008; Tran *et al.*, 2006]. This is in contrast to the more-well studied TNF-αR system where a number

of studies show a transient NF-κB activation in response to sustained TNF-α stimulation [Lee *et al.*, 2000, 2014; Maecker *et al.*, 2005; Quivy *et al.*, 2002; Rogers and Fuseler, 2007; Saitoh *et al.*, 2003].

In our previous work [Khetan and Barua, 2019] we unidentified two uniqe interactions in the Fn14 pathway which could give rise chronic Fn14 overexpression. Firstly, Fn14 can activate without requiring stimulation by TWEAK when it is overexpressed on the cell surface [Brown *et al.*, 2013; Winkles, 2008]. Secondly, Fn14 induced nuclear localization of NF-κB can itself induce the expression of the Fn14 gene and de novo synthesis of the protein [Tran *et al.*, 2006]. Thus, it can potentially give rise to positive feedback regulation in the system [Kwon *et al.*, 2014; Tran *et al.*, 2006]. We may hypothesize that a cell when stimulated with TWEAK activates the NF-κB pathway resulting in Fn14 expression. This may result in Fn14 overcrowing on the plasma membrane which may allow the signal to be sustained without requiring TWEAK stimulation.

The NF-κB pathway is most extensively studied by activation of the TNFαR receptor by TNFα. Upon activation of the pathway by TNFα, kinases act upon the cytoplasmic complex of NF-κB and its inhibitor IκBα. The IκBα is then degraded thereby allowing NF-κB to rapidly translocate to the nucleus where it binds to specific sites on the genome and triggers transcription of various genes [Brasier, 2006]. Over the past two decades a number of detailed mathematical models for this pathway have been developed [Basak *et al.*, 2007; Cheong *et al.*, 2008; Hoffmann *et al.*, 2002, 2006; Kearns *et al.*, 2006; O'Dea *et al.*, 2007]. The most recent model resulting from the culmination of the previously mentioned work is by Paszek et al [Paszek *et al.*, 2010] which uses a semi-stochastic method and incorporates cell to cell variation. These models however deal exclusively with the TNF-αR system, with the implication being that since other members of the TNF superfamily behave similarly, these models could act as satisfactory proxies for studying their signaling dynamics as well. However, with the discovery of certain unique aspects of TWEAK-Fn14 signaling such as ligand independent activation [Brown *et al.*, 2013; Winkles, 2008], Fn14 de novo

synthesis [Tran *et al.*, 2006] and strong non-canonical pathway activation [Burkly, 2015], the Paszek model may not be sufficient to explain the peculiar experimental behavior of TWEAK-Fn14. Given the implications of Fn14 signaling in very lethal pathologies, it has become necessary to develop a computational model which incorporates the most up-to-date evidence of Fn14 signaling behavior. Here we present a comprehensive TWEAK-Fn14 signaling model which accounts for the above mentioned aspects of the system and provides a platform for future in-silico experiments.

2. METHODS

2.1. CELL CULTURE

MDAMB-231 cells were grown in RPMI 1640 medium (Corning Life Sciences) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Gibco). Approximately 100,000 cells were grown in each well of a 24 well plate. Cells were treated with 100 ng/mL of recombinant human TWEAK (Peprotech cat. no. 310-06) for various durations upto 12 hours before further analysis.

2.2. FLUORESCENCE MICROSCOPY

Whole cell staining of cellular proteins was performed by culturing cells as described before fixing and permeabilizing using the Image-iT Fix/Perm kit (Invitrogen) using the manufacturer's protocol. Cells were then blocked with 3% Bovine serum albumin in phosphate buffered saline. Fn14 proteins were stained with mouse IgG Item-4 antibody (eBioscience) followed by anti-mouse FITC conjugates secondary antibody. NF-κB subunits were stained using either anti-p65 or anti-p50 primary antibody followed by FITC or PE conjugated secondary antibody. After sufficient washing, cells were analyzed under a fluorescence microscope. Staining of only membrane bound Fn14 was accomplished with the same procedure but without permeabilization.

2.3. FLOW-CYTOMETRY

For surface Fn14 quantification, cells were grown as above, detached with trypsin and immediately cooled on melting ice to arrest biological processes. This was followed by fixation and staining with antibodies as described above. Cell membrane integrity was determined by co-staining with propidium iodide (eBioscience). Cells were then analysed using a flow cytometer (BD Accuri C6 Plus).

2.4. COMPUTATIONAL MODEL

We developed a Fn14-NF-κB signaling pathway model based on our previous work [Khetan and Barua, 2019]. The model describes biochemical interactions and transformations of a list of signaling protein molecules in the Fn14-NF-κB signaling pathway. In addition to the various novel protein interactions introduced in our previous work, such as Fn14 trimerization, Fn14 transcription etc. [Khetan and Barua, 2019], we now include Fn14 receptor trafficing to the early endosome (with and without ligand recruitment), Fn14 recycling to the surface and activation of the non-canonical NF-κB pathway based on reaction rules published by Basak et al [Basak *et al.*, 2007] and Shih et al [Shih *et al.*, 2009]. The model was defined in BNGL [Blinov *et al.*, 2004; Faeder *et al.*, 2009]. BNGL allows one to define course-grained features of proteins such as binding sites and motifs. The interactions between these proteins are governed by simple reaction rules with specified rate constants. Based on these species and reaction rules, a list of potential species is generated each with as associated ordinary differential equation governing its concentration [Faeder *et al.*, 2005]. The model was executed using the software BioNetGen [Harris *et al.*, 2016]. BioNetGen evaluates BNGL rules and calculates all possible species and reactions that can generate from the site-specific interactions and transformations of the molecules in a model.

2.5. SIMULATION

Simulations were carried out using BioNetGen. The software generates ODEs describing the mass conservation of species in the network of a BNGL-defined model. It also performs simulation using the numerical ODE solver package CVODE [Cohen *et al.*, 1996].

3. RESULTS AND DISCUSSION

3.1. TWEAK INDUCES RAPID FN14 INTERNALIZATION

Previous studies suggest that only a fraction of Fn14 is usually available on the cell surface [Meighan-Mantha *et al.*, 1999]. This could be in part due to the necessity to keep Fn14 surface concentrations low in order to prevent self-trimerization and maintain the cell in a non-active basal state. Fluorescence microscopy of unstimulated MDAMB-231 cells with immunostained Fn14 reveals that indeed most of the Fn14 is internalized into cytoplasmic components. (Figure 1 A). This preliminary finding was significant as it suggested that intracellular trafficking of the Fn14 receptor may be a significant contributor to TWEAK-Fn14 signaling dynamics and therefore cannot be overlooked. On further investigation, we found that not only is most Fn14 not found on the surface, but that Fn14 is rapidly internalized upon TWEAK stimulation (Figure 1 B&C). We incubated cells with an anti-human Fn14 primary antibody and then stimulated the cells with TWEAK. The internalized Fn14 – being labeled with the primary antibody recruited at the surface – was then stained with a FITC labeled secondary antibody following fixation and permeabilization. Figure 1 C shows that following TWEAK stimulation for 30 minutes, Fn14 is clearly trafficked to intracellular compartments, significantly reducing the Fn14 available at the surface for TWEAK recruitment. Such receptor trafficking behavior could significantly affect the TWEAK-dependent pathway activation dynamics.

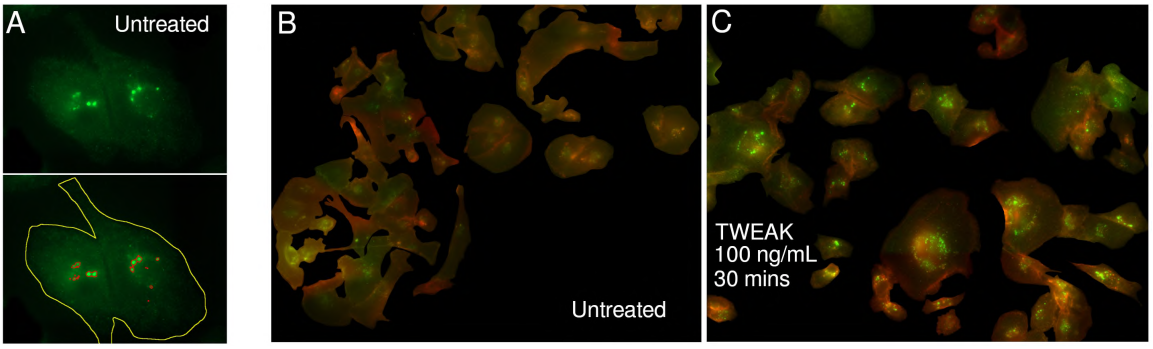

Figure 1. Fn14 distribution in unstimulated and TWEAK treated cells. *A*, MDAMB-231 cells were fixed, permeabilized and immunostained with anti-Fn14-FITC. Most Fn14 protein is localized to cytoplasmic vesicles (red) as opposed to on the cell surface (yellow). *B & C*, Live cells were incubated for 15 mins with anti-Fn14 primary antibody, washed, then treated with 100 ng/mL of TWEAK for 30 mins. Cells were then fixed and stained with anti-Fn14-FITC secondary antibody and DiO plasma membrane dye without permeabilization. B and C show cells with or without TWEAK treatment.

We then investigated the rate of Fn14 internalization in response to TWEAK stimulus. In order to quantify the TWEAK induced internalization rate, cells were treated with increasing durations of TWEAK, followed by detachment, cell-surface immunostaining for Fn14 and flow-cytometry analysis. Since the cells were not permeabilized, only membrane bound Fn14 was stained while internalized Fn14 remained unstained. Exclusion of membrane permeable cells was further insured by propidium iodide (PI) counter staining and then gating the PI-positive cells out of the analysed data. Figure 2 shows the results from flow-cytometry analysis. The left panel shows representative violin plots of plasma membrane-bound Fn14 fluorescence in cells treated with TWEAK for 0, 5, 10, 20, 30, 45 and 60 mintes. Even 5 minutes of TWEAK stimulation is sufficient to induce a significant reduction in FITC fluorescence indicating a rapid internalization of membrane-Fn14. However, TWEAK, being an inducer of apoptosis, causes significant *in vitro* cytotoxicity beyond 45 minutes of stimulation (Supplementary Figure B.1.D, Appendix B). This led us to restrict model fitting to 45 minutes, when most cells were still healthy. The right panel of Figure 2 shows the average fluorescence of 3 experiments of cells treated with TWEAK 100 ng/mL

for 0, 5, 10, 20, 30 and 45 minutes. The sharp decline in surface fluorescence indicates rapid internalization to a stable minimum of membrane-Fn14 in just 10 minutes. Since this behavior is not seen in TNFα and other superfamily members [Algeciras-Schimnich *et al.*, 2002], we identified ligand induced rapid internalization as another unique feature of the TWEAK-Fn14 system.

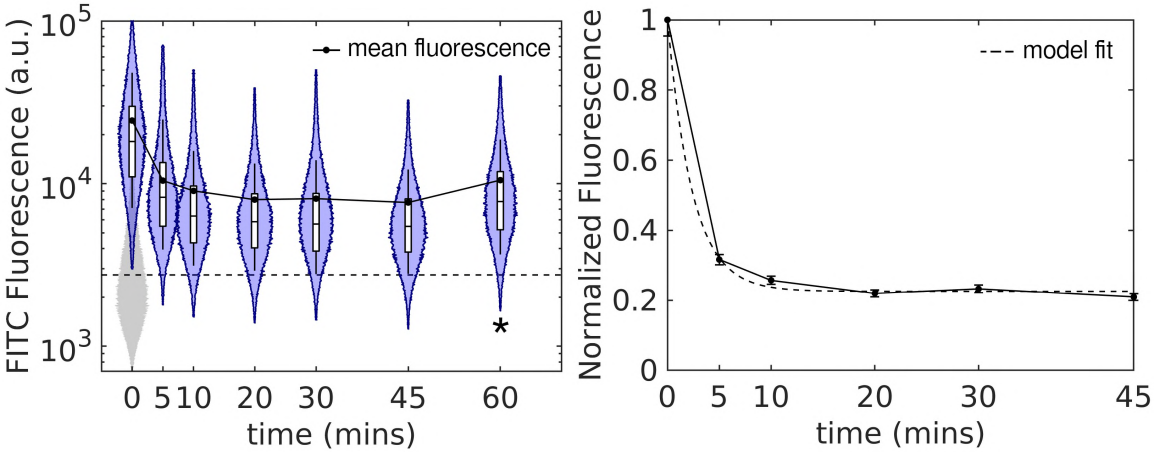

Figure 2. Flow-cytometry analysis of Fn14 internalization. MDAMB-231 cells were treated with 100 ng/mL of TWEAK for the indicated times. Cells were then detached and stained with anti-Fn14 and FITC labeled antibodies without permeabilization and analyzed via flow-cytometry. Permeabilized cells were excluded via PI staining. Left panel shows FITC fluorescence histograms as violin plots for 0 to 60 minutes of TWEAK treatment. Box plots delineate the 0.25 and 0.75 quantiles. Right panel shows averages fluorescence of 3 experiments with >50,000 cells each. Model fit of receptor internalization is shown with a dashed line.

**The increase in fluorescence at 60 minutes coincides with a substantial increase in TWEAK induced cytotoxicity (Supplementary Figure B.1.D), making the plotted data a representation of only living cells which do not represent the entire cell population. We therefore decided to perform model fitting only up till the time when cytotoxicity is negligible, that is till 45 minutes. This model limitation is further discussed in the Conclusion.*

3.2. TWEAK INDUCED FN14 INTERNALIZATION IS NOT ASSOCIATED WITH DEGRADATION

The rapid internalization of Fn14 receptors due to TWEAK stimulation naturally leads to the question of the receptors' ultimate fate once inside the cell. In previous studies of other TNFα super-family members, ligand induced internalization is often followed by lysosomal degradation [Higuchi and Aggarwal, 1994; Tsujimoto and Vilcek, 1987] or receptor recycling to the plasma membrane. To see if the same can be observed for Fn14, we analyzed the location of internalized Fn14 by fluorescence microscopy. Cells were incubated with anti-human Fn14 primary antibody for 15 minutes followed by washing and further incubation with Lysotracker Deep Red and TWEAK for 45 minutes. Cells were then fixed, permeabilized and immunostained with anti-FITC secondary antibody (Figure 3, left). The Lysotracker dye is a pH responsive dye designed to stain acidified late-endosomal and lysosomal compartments. Trafficking of proteins to these vesicles results in enzymatic protein degradation. Contrary to expectations, we see that even after 45 minutes of TWEAK stimulation, the green (Fn14) and red stained regions (lysosomes) are not co-located.

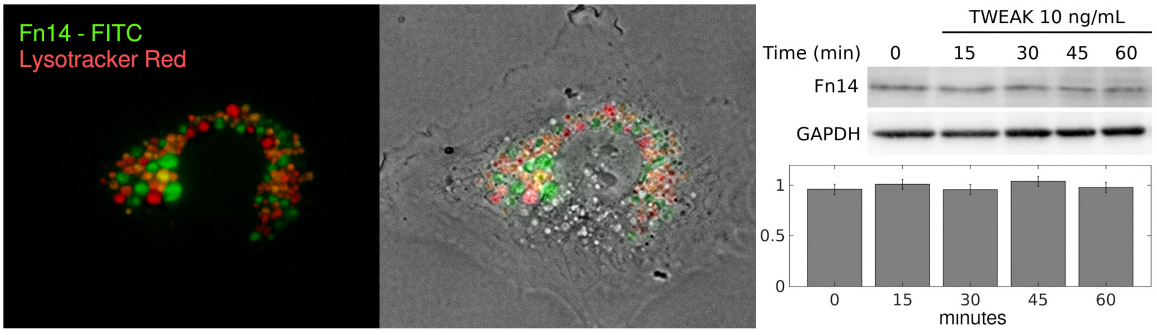

Figure 3. Intracellular fate of Fn14. Cells were treated together with 100 ng/mL of TWEAK and Lysotracker dye for 45 mins and then fixed, permeabilized and immunostained with anti-Fn14-FITC (green). The right panels show western-blot analysis to total Fn14 levels after TWEAK stimulation 0, 15, 30, 45, and 60 minutes. The western blot quantification is shown in the bar chart. Fn14 expression was normalized against β-actin. Each bar shows and average of 3 experiments with 10^5 cells each.

This indicates that although Fn14 is strongly internalized, the vesicles containing the internalized Fn14 do not progress to late endosomes or lysosomes. This provides evidence that following ligand mediated endocytosis, Fn14 is stored in the early endosome for eventual recycling to the plasma membrane. Furthermore, we quantified total Fn14 concentration in cells treated with TWEAK for upto 60 minutes via western-blot analysis. Figure 3B shows that the total Fn14 protein content is not significant altered in response to TWEAK stimulation of two different doses. We take these two findings – the lack of lysosomal trafficking of Fn14 and constant total Fn14 levels – as evidence that TWEAK does not induce any significant expression or degradation of cellular Fn14 levels in MDAMB-231 cells. In other words, we infer that any signaling behavior dependent on Fn14 availability my be attributed to the trafficking of Fn14 to and from the plasma membrane alone.

3.3. TWEAK MAINTAINS LONG-TERM CANONICAL NF-κB SIGNALING

We now turned our focus to the downstream effects of TWEAK-Fn14 signaling, namely the activation of the NF-κB pathway. Localization of the NF-κB family of dimers into the nucleus is mediated by two distinct but somewhat interrelated pathways: the canonical and non-canonical pathways. The canonical pathway results in the nuclear localization of the p65 unit of the NF-κB dimer whereas the non-canonical pathway results in nuclear localization of the p50 unit. Figure 4 shows the nuclear localization of the p65 subunit of NF-κB. MDAMB-231 cells were stimulated with 100 ng/mL of TWEAK followed by fixation, permeabilization and immunostaining with anti-human p65 labeled with FITC. The top panel of Figure 4 shows p65 primarily localized in the cytoplasm when not stimulated with TWEAK. The bottom panel shows that a significant fraction of cytoplasmic p65 has been transported to the nucleus following TWEAK stimulation. This nuclear localization of NF-κB proteins is a measure of pathway activation and was quantified by the correlation of green and blue pixel intensities (Figure 4, right) as explained below..

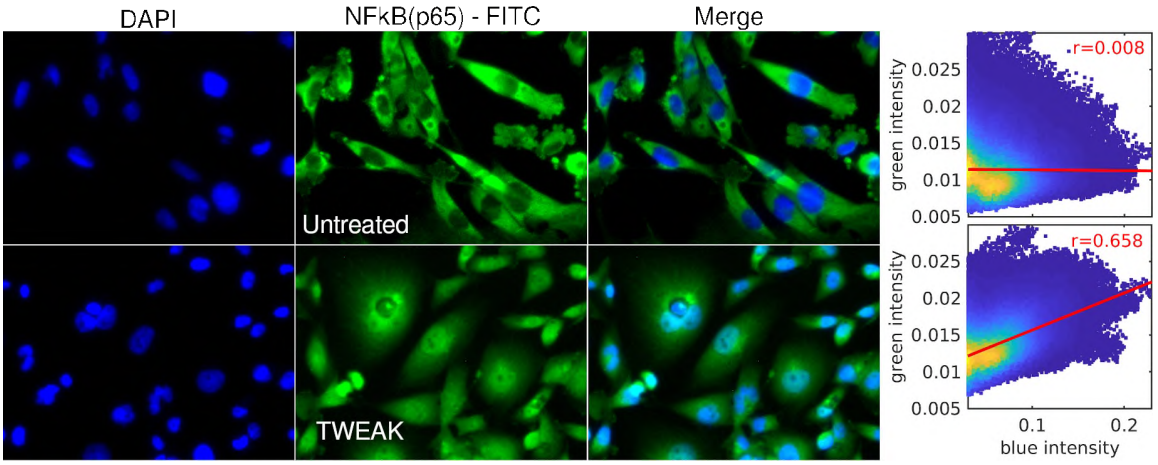

Figure 4. Nuclear trafficking of NF-κB subunit p65. Cells were treated with 100 ng/mL of TWEAK for 30 mins and fixed, permeabilized, and immunostained with anti-p65-FITC and DAPI. Top and bottom panels show representative cells with or without TWEAK stimulation. The right panels show scatter plots of green vs. blue pixel intensities and the respective blue-green correlation coefficients. A low correlation coefficient (top) indicates low nuclear localization of p65.

Image analysis of NF-κB trafficking was performed using a three step process. The three steps are represented by panels A, B and C in Figure 5. Step A: First, cells were treated with varying durations of TWEAK upto 6 hours. Then cells were immunostained for p65-FITC (green) as well as p50-PE (red). Images were taken of large regions containing approximately 1,000 cells each (Figure 5A). Step B: The green (or red in the case of the non-canonical NF-κB subunit, p50) value of each pixel was plotted against its blue value (DAPI, nuclear stain) on a scatter plot. The degree of correlation between green and blue regions was used as a measure of NF-κB nuclear localization. This was quantified by calculating the correlation coefficient of the green-blue values of each pixel (Figure 4 & Figure 5B). Step C: The correlation coefficient for the green-blue values of each pixel was then plotted for each TWEAK duration. The correlation coefficients were normalized such that the maximum nuclear localization was quantified as 1 (Figure 5C). The same steps were performed for the p50 NF-κB labeled with PE (red). Our data shows that the canonical NF-κB pathway (quantified by p65 nuclear localization) is rapidly activated in response

to TWEAK stimulus and remains active for upto 6 hours. In contrast, the non-canonical pathway (quantified by p50 nuclear localization) is activated slowly and is not a significant contributor to overall nuclear NF-κB levels even after 6 hours of TWEAK stimulation. This seems to confirm our earlier finding that long-term NF-κB activation does not necessarily require the activation of the non-canonical pathway and can be explained by canonical pathway activation alone [Khetan and Barua, 2019].

3.4. EVALUATION OF POTENTIAL THERAPIES USING AN UPDATED TWEAK-FN14-NF-κB MODEL

An ordinary differential equation based computational model for the TWEAK-Fn14 signaling system was first published by our group for the first time in 2019 [Khetan and Barua, 2019]. This model was based on the TNFα-NF-κB model published by Tay et al. [Tay *et al.*, 2010] with the addition of reaction rules governing the unique trimerization and constitutive activation behavior of Fn14. Using experimental data gathered in the present study for ligand independent Fn14 receptor distribution, ligand mediated Fn14 receptor trafficking and the nuclear localization of the canonical and non-canonical NF-κB subunits, we updated our model with the addition of relevant reaction rules governing these new findings. A complete list reaction rules, parameters and species can be found in Appendix A of this dissertation. The updates to the model can be briefly summarized as follows. We first chose Fn14 internalization (k_{int}) and recycling rates (k_{rec}) such that an unstimulated cell at steady state has approximately 10% of its receptors on the plasma membrane while the remaining 90% is maintained in the early endosomal compartment (surface to internalized Fn14 ratio based on fluorescence data from Figure 1A). A small fraction of the latter is degraded (reaction rate d_f) while a small amount of cytoplasmic Fn14 is synthesized (reaction rate $c_{4,f}$) in order to maintain Fn14 homeostasis. The ligand dependent internalization ($k_{int,t}$) is kept much higher than ligand-independent internalization (k_{int}) such that the membrane bound Fn14 levels to fit the data in Figure 2. Reaction rates

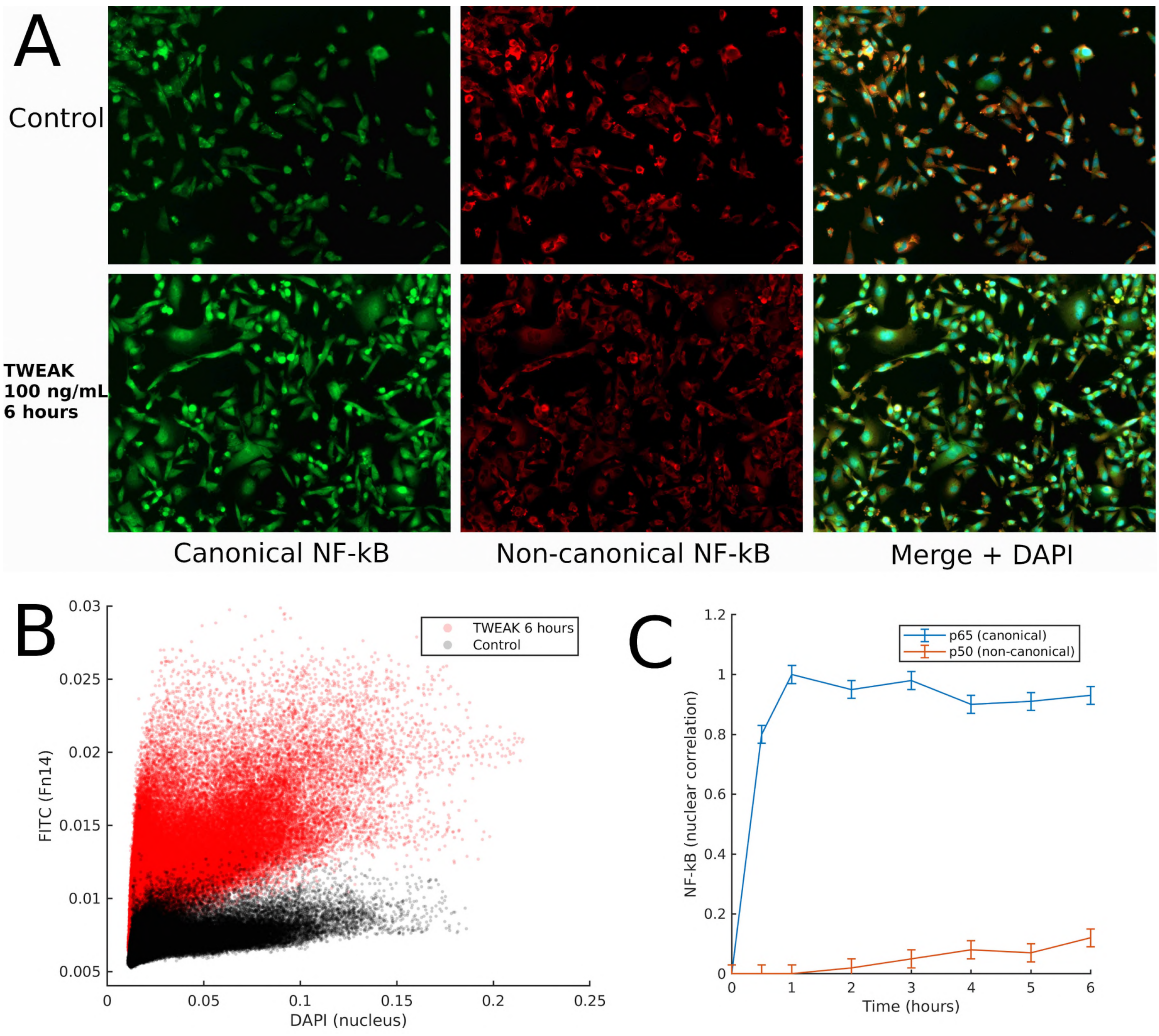

Figure 5. Quantification of nuclear localization of NF-κB though image processing. *A*, Fluorescence microscopy images of cells with and without TWEAK treatment. Cells were fixed, permeabilized and stained for p65(green) and p50 (red). The right most panels show merged channels along with DAPI nuclear stain (not shown separately). *B*, Green and blue intensity values are plotted for each pixel. The degree of linearity of the data is a measure of the nuclear localization of p65 proteins. Panel B shows representative data from untreated (control) and 6 hour TWEAK stimulated MDAMB-231 cells. *C*, Normalized NF-κB nuclear correlation values are plotted for TWEAK stimulation upto 6 hours.

of non-canonical NF-κB signal transduction steps have been described by Shih et al. [Shih *et al.*, 2012]. Briefly, their model involves signal transduction based on the p100/IκBδ-NF-κB cytoplasmic complex. Upon stimulation, the IκBδ protein is phosphorylated and then degraded leaving the cytoplasmic NF-κB dimers to translocate to the nucleus. These

reactions rule were incorporated into our model without much modification except for the change in IκBδ phosphorylation rate by fitting the model to the data in Figure 5C (orange curve). The degradation rate of phosphorylated IκBδ and the nuclear transport rate of p50 were assumed to be the same as the corresponding reactions in the canonical pathway owing to similar size and structure of the proteins involved.

We now created two kinds of 'cells', differentiated solely in their plasma membrane Fn14 expression. The type 1 cell has low Fn14 expression on the plasma membrane (only 10%, as explained above) and represents normal, disease-free cells in our body. The type 2 cell has a high fraction of Fn14 on the plasma membrane, 30% (quantified from cervical carcinoma HeLa cells [Gurunathan *et al.*, 2014], Supplementary Figure B.2, Appendix B) and represents Fn14 overexpressing tumor cells such as those of glioblastoma, melanoma, metastatic cervical and lung carcinomas etc. The latter type of cell also shows persistent NF-κB activation. Our previous study [Khetan and Barua, 2019] suggested that, owing to the Fn14 constitutive activation positive feedback loop, these two types of cells may be descriptions of two stable states within the same cell; a stable inactive state and an oscillatory activated state. The cell may be able to switch 'on' or 'off' depending on various stimuli. Consequently, it may also be possible to target the pathway using a variety of signal attenuating therapies which may 'switch-off' a pathologically activated cell. We test three such potential therapies on our comprehensive model. Two of these therapies are familiar to medical research: Fn14 trimer antagonist (Enavatuzumab, [Chao *et al.*, 2013; Lam *et al.*, 2018; Ye *et al.*, 2017]) and anti-Fn14 siRNA [Peng *et al.*, 2018; Watts *et al.*, 2007], while a third therapy, targeted lysosomal degradation [Banik *et al.*, 2020], is novel. Each 'therapy' is simulated by targeting a particular reaction step and multiplying the reaction rate constant by a constant λ. Thus, if $\lambda = 10$, it implies that the particular reaction rate constant has been increased ten-fold.

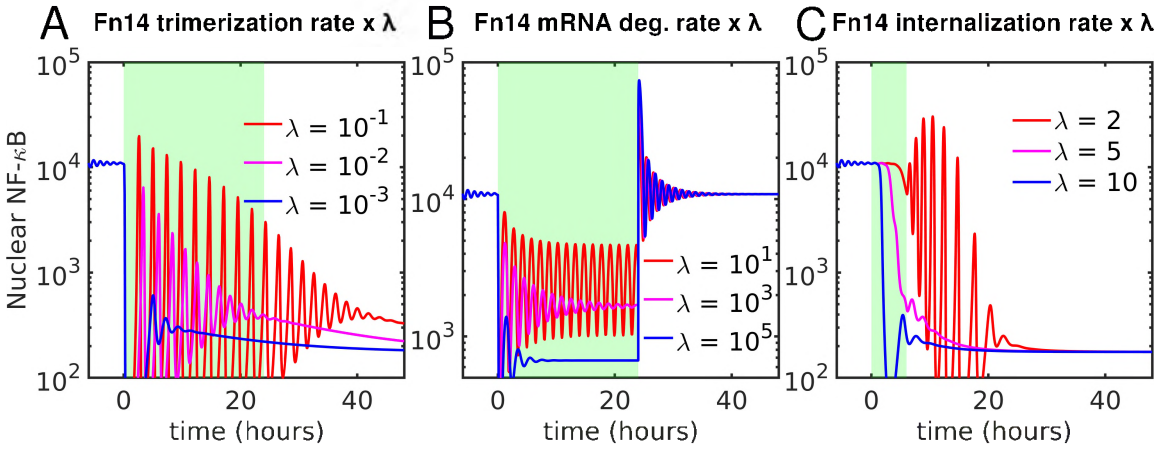

Figure 6. Single cell response dynamics to targeted treatments. A single Fn14-upregulated cell was subjected to three kinds of pathway-attenuating treatments of varying intensity. In each case, a particular protein reaction rate was increased or decreased by a factor of λ. The duration of treatment is indicated by the green shaded region. Cellular response is shown by plotting nuclear NF-κB levels. *A*, Fn14 antagonist treatment is simulated by a reduction in Fn14 trimerization rate, $k_c \cdot \lambda$, for 24 hours followed by return to original conditions. *B* siRNA treatment is simulated by increase in mRNA degradation rate, $c_{4,fn14} \cdot \lambda$, for 24 hours followed by return to original conditions. *C*, LYTAC treatment is simulated by an increase in Fn14 internalization rate, $k_{int} \cdot \lambda$, for 6 hours followed by return to original conditions.

3.4.1. Therapy A: Antagonist Mediated Prevention of Fn14 Trimerization. As discussed previously, TWEAK-dependent and TWEAK-independent trimerization of Fn14 is the critical first step in pathway activation. Bifurcation analysis conducted by Khetan et al. [Khetan and Barua, 2019] suggests that a drop in Fn14 trimerization may be able to 'switch off' a constitutively activated cell. To investigate this, we subjected a type 2 (constitutively active) cell to anti-trimerization treatment by reducing the Fn14 trimerization rate, k_c by λ (Reaction rules 3 & 4). The new rate constant was $k_c \cdot \lambda$ where $\lambda = 10^{-1}$, 10^{-2} and 10^{-3} (Figure 6A). $\lambda = 10^{-3}$ results in the sharpest decline in Fn14 trimerization and hence represents the most intense antagonist effect. The 'treatment' lasted for 24 hours followed by a return to the basal trimerization rate. Figure 6A shows that treatment results in a gradual oscillatory decline in nuclear NF-κB levels to the basal inactive state. Furthermore, the cell does not return to the previous constitutively active state upon cessation of therapy.

3.4.2. Therapy B: Anti-Fn14 siRNA. Another crucial contributor of the Fn14 positive feedback loop has been identified as the NF-κB induced expression of Fn14. Thus it seemed intuitive to test a potential siRNA based therapy to attenuate the feedback loop. SiRNA's mediate the RNA interference pathway by binding to mRNA's with complementary sequences and causing their degradation prior to translation into proteins. Here we simulate anti-Fn14 siRNA treatment by increasing the Fn14 mRNA degradation rate, $c_{3,fn14}$ by a factor of λ, where $\lambda = 10$, 10^3 or 10^5. Figure 6B shows that siRNA treatment is capable of lowering the nuclear NF-κB levels to a lower oscillatory state but this effect is transient and the levels return to the previous levels after cessation of siRNA treatment. It is interesting to note that even when the siRNA based attenuation is most intense, i.e. when the mRNA degradation rate is increased by an order of 5, the nuclear NF-κB levels return to their activated state after siRNA treatment subsides. This is primarily due to the fact that siRNA prevents the expression of new Fn14 proteins but has no significant impact on Fn14 levels already present in the cell. Since, we know that cellular Fn14 levels are largely stable over time (Figure 3), the amount of Fn14 remaining in the cells could be sufficient to maintain signal activation in therapeutically relevant timescales.

3.4.3. Therapy C: Targeted Lysosomal Trafficking of Membrane-Fn14. A major finding of the present study has been the rapid internalization of Fn14 in response to TWEAK stimulus in cells that are not constitutively active. This behavior is a significant contributor to the cell's ability to return to the basal inactive state by reducing the number of surface Fn14 receptors capable of partaking in signal activation. In fact we were able to create a constitutively active cell (type 2 cell) simply by reducing the internalization rate to keep approximately 30% of the Fn14 on the surface. We see in tumors such as glioblastoma that overexpress surface Fn14 [Tran *et al.*, 2006, 2005], that TWEAK does not trigger any significant internalization. We therefore hypothesized that targeted internalization and lysosomal degradation of the surface Fn14 could be potential therapeutic approach. Such an approach would accomplish two tasks: (a) reduce surface Fn14 which initiates signaling

and (b) reduce the overall amount of Fn14 already present in the cell, something that is not possible with siRNA therapy. This can be accomplished by employing a newly developed technology named lysosome-targeting chimaeras (LYTAC) by Banik et al. [Banik *et al.*, 2020]. Here, a protein targeting antibody is fused with lysosomal trafficking glycopeptides to form a 'chimera'. When cells are treated with these chimeras, the target surface protein is rapidly internalized and then trafficked to the lysosome for degradation. We simulated such a treatment by simply increasing Fn14 trimer internalization rate, k_{int} by a factor λ, where $\lambda = 2$, 5 or 10. Figure 6C shows that even with 6 hours of LYTAC treatment, the nuclear NF-κB levels drop rapidly to the inactive basal state level. Even a modest two-fold increase in the internalization rate results in long lasting NF-κB suppression after treatment has subsided. This suggests a new and potentially significant method of 'switching-off' Fn14-overactive cells.

4. CONCLUSION

TWEAK-dependent and TWEAK-independent Fn14 trafficking to and from the plasma membrane was studied by fluorescence microscopy. We found that TWEAK mediated Fn14 internalization is a critical self limiting mechanism employed by the cell to maintain low NF-κB levels and prevent the cell from 'switching' to the constitutively active state. We also studied the cross-talk between canonical and non-canonical NF-κB activation quantifying the nuclear localization of the p64 and p50 subunits of NF-κB. We found that non-canonical NF-κB is only a small contributor the overall nuclear NF-κB levels and that the canonical pathway is sufficient to account for long-term NF-κB activation in response to TWEAK stimulus. For the first time, receptor internalization, receptor recycling and non-canonical NF-κB activation was included in a comprehensive TWEAK-Fn14-NF-κB pathway computational model using reaction rules written with BioNetGen. A significant shortcoming of the present model however is that it does not account for ligand induced cell death and its effect on the population level. TWEAK, being an inducer of apoptosis

via multiple pathways, was seen to induce significant toxicity beyond 45 minutes of *in vitro* stimulation (Supplementary Figure B.1.D). In this case, removal of a large number of cells due to cell death may shift the population average protein levels (such as surface Fn14, Figure 2A, 60 minutes) in ways that cannot yet be accounted for by the model. For instance, if a hypothetical subpopulation of cells were insensitive to TWEAK induced apoptosis, the present model could not explain the surface Fn14 levels in those cells. In the absence of apoptotic pathways in the model, we decided to fit the model up till the point where toxicity is still minimal. On the other hand, a more comprehensive model that includes TWEAK induced apoptotic pathways may be developed to simulate that only a high surface Fn14 expressing subpopulation remains after most cells have been killed by 60 minutes of TWEAK. Ou future work on TWEAK-Fn14 modeling should include TWEAK induced apoptosis and cell-to-cell variation, which are lacking in the current model.

Finally, this updated model was used to create two types of cells: (a) low-Fn14, low NF-κB activity and (b) high-Fn14 and constitutively active NF-κB cells by simply altering the steady-state surface Fn14 levels. Type 2 cells were used as a model for Fn14 overexpressing tumor cells such as those of glioblastoma etc., and three potential therapeutic approaches were tested *in silico*. We found that intuitive approaches such as Fn14 antagonist therapy or Fn14 siRNA therapy provided at best a modest ability to 'switch off' constitutive activity in the cell. However, a novel therapy involving targeted lysosomal degradation of surface Fn14 showed a drastic and long-lasting reduction in NF-κB activity after a relatively short treatment. This study for the first time provides a computational model which is capable of accounting for a wide range of unique experimental observations of the TWEAK-Fn14 signaling system and provides a useful platform for *in silico* experiments of various protein targets in the pathway.

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SECTION

2. CONCLUSIONS

2.1. SUMMARY

2.1.1. Unique Fn14 Behavior Results in Positive Feedback Loop. Our model predicts that the combination of two unique features of Fn14: (a) ligand independent trimerization and (b) NF-κB induced Fn14 expression are necessary and sufficient to create a positive feedback loop where activation of the pathway results in Fn14 expression which in turn causes crowding of Fn14 receptors on the plasma membrane which may then cause constitutive trimerization and ligand-independent activation. Such behavior has been hypothesized but not studied thoroughly via a mechanistic model prior to this study.

2.1.2. System Bistability Leads to Switching Behavior. The induction of the positive feedback loop results in highly non-linear system dynamics including a wide bistable region over a range of two crucial parameters. The system is bistable between a constitutively active oscillatory state and a stable inactive state. This allows us to predict that cells may employ such dynamics to switch 'on' or 'off' pathway activation in response to the intensity and duration of pro-NF-κB stimuli. This also suggests that it may be possible to 'switch off' a pathologically active cell by targeting key proteins in the pathway.

2.1.3. Persistent NF-κB Requires Certain Key Features. Our findings suggest that, contrary to previous assumptions, the non-canonical NF-κB pathway may not be necessary to explain long term activation of NF-κB in response to TWEAK. We find that a positive feedback loop in the canonical pathway alone is both necessary and sufficient to result in persistent nuclear localization of NF-κB. Furthermore, fluorescence microscopy analysis of non-canonical NF-κB distribution reveals only a minuscule amount of non-canonical NF-κB p50 protein localization in the nucleus after long lasting TWEAK stimulation.

2.1.4. Receptor Trafficking is Key Contributor to System Dynamics. Fn14 distribution in unstimulated and TWEAK-stimulated cells shows that rapid internalization of Fn14 is a crucial feature of this signaling pathway. Internalization prevents further signal transduction and prevents a cell from accidentally 'switching' to the pathologically active state in response to insignificant stimulus.

2.1.5. An Evaluation of Potential Anti-Fn14 Therapies. *In silico* tests of three potential anti-Fn14 therapies reveals that familiar and intuitive interventions such as Fn14 antagonists and anti-Fn14 siRNA provide modest or short-lived benefits. However, a novel targeted Fn14 degradation therapy provides rapid and long lasting attenuation of pathway activation.

2.2. RECOMMENDATIONS FOR FUTURE WORK

TWEAK being a potent inducer of apoptosis in cancerous and non-cancerous cells Chicheportiche *et al.* [1997], was seen to cause cytotoxicity during long *in vitro* incubation. This has the potential to artificially skew protein quantification data towards only those cells that survive TWEAK induced apoptosis. In the absence of apoptotic pathways in the model, it became necessary to fit the model up till the point where toxicity is still minimal. This points to strong need for a comprehensive model that includes TWEAK induced apoptotic pathways which may simulate the behavior of cells over a whole range of their susceptibility to TWEAK induced apoptosis. Our future work on TWEAK-Fn14 modeling should include TWEAK induced apoptosis and cell-to-cell variation, which are lacking in the current model.

The question of Fn14 mediated activation of the non-canonical NF-κB and crosstalk between canonical and non-canonical pathways remains largely unanswered. It is recommended to perform detailed single-cell level study of downstream proteins of each pathway using methods employed in Paper II of this dissertation; namely intracellular distribution via microscopy and cell-to-cell variation via flow-cytometry. In particular, since TWEAK

is a known inducer of apoptosis, This will allow the model to be refined and eliminate a significant number of unknown reaction rate parameters. Furthermore, it is yet to be determined whether the human Fn14 gene promoter region has non-canonical NF-κB binding sites (binding sites have already been discovered for the canonical NF-κB by Tran et al. [Tran *et al.*, 2006]). If indeed such sites exist, it implies that non-canonical NF-κB too is capable of inducing Fn14 transcription, potentially giving rise to another positive feedback loop in the signaling pathway.

Of the various anti-Fn14 therapies which have been described in literature, most have not been investigated in terms of their underlying molecular effects. For example, the mechanisms by which Enavatuzumab, a humanized Fn14 antagonist, attenuates Fn14-NF-κB activity are largely not understood. It is recommended that such therapies be evaluated in the context of the present computational model in order to provide a more rational path to safe and effective anti-Fn14 therapies.

Lastly, therapeutic approaches suggested in this study have not been experimentally verified. It is recommended that antagonist therapy, siRNA therapy and LYTAC therapy be performed on pathologically active tumor cells. In addition to their overall pathway attenuating abilities, it is necessary to determine the molecular effects on the various downstream proteins in response to each therapy.

APPENDIX A.

COMPUTATIONAL MODEL

1. BIONETGEN RULES

Below we provide BNGL rules describing the protein-protein interactions and biochemical transformations in the model. These rules can be broadly divided into the following steps: 1) Fn14 trimerization in the plasma membrane, 2) activation of the NF-κB pathway and other downstream events. In our model, most of the molecular interactions and reactions associated with Step 2 are taken from Tay et al. [Tay *et al.*, 2010].

1.1. FN14 TRIMERIZATION IN THE PLASMA MEMBRANE

This step occurring in the cell plasma membrane deserves a more detailed explanation because it is still not very well-characterized experimentally. We make certain assumptions based on the available knowledge. We consider both TWEAK-induced and constitutive Fn14 trimerization, as illustrated in Figure A.1 and Figure A.2.

TWEAK naturally remains as trimers in a solution. In the model, we represent such trimers by a single ligand molecule with three identical binding sites for Fn14 (Figure A.2). The ligand can first be recruited from solution to form a 1:1 ligand-receptor complex. Subsequently, the remaining two unoccupied sites of the ligand can sequentially engage more Fn14 to form 1:2 and 1:3 complexes (Figure A.2).

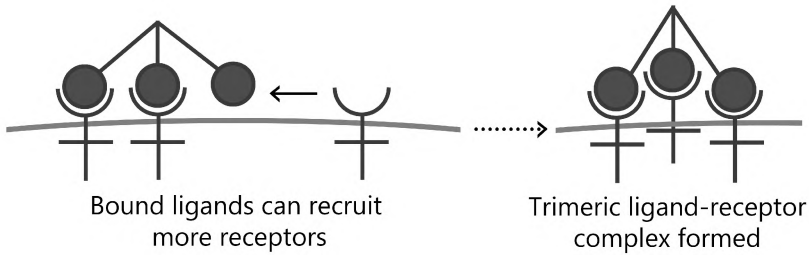

Figure A.1. TWEAK-mediated Fn14 trimerization. The trimeric form of TWEAK (three black circles) can sequentially engage up to three Fn14 molecules in a complex.

The constitutive trimerization requires each receptor molecule to have at least two binding sites (Figure A.2). This allows the protein to form homodimers as an intermediate complex. Moreover, because of the two sites, we consider the possibility of a ring structure, as shown (Figure A.2). Once a linear trimer is formed, it undergoes a fast ring-closure step to create the ring structure. In the model, we restrict maximum three receptors per complex, thus prohibiting tetramer or larger complex formation via this constitutive interaction.

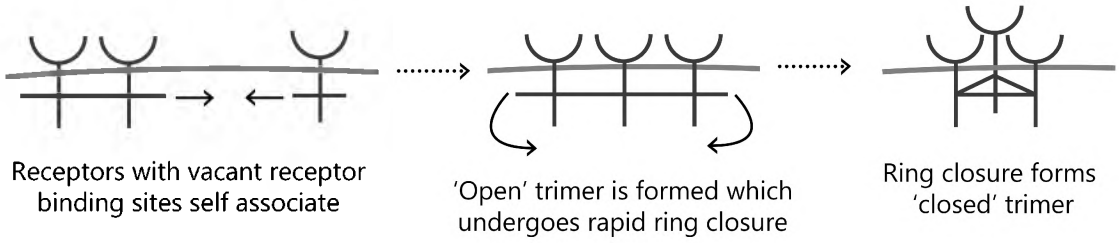

Figure A.2. Fn14 trimerization via constitutive self-assembly. Each Fn14 contains two lateral 'arms' to self-assemble with other Fn14 molecules. The reaction can generate various chain and ring structures containing up to three receptor molecules in a complex.

In BNGL, we define the trimeric form of TWEAK as `TWEAK(r,r,r)`, where each `r` represents one receptor binding site. We define Fn14 as `Fn14(l,r,r)`, where `l` represents its sole TWEAK binding site and `r` represents two identical sites for constitutive self-association.

Rule 1: This BNGL rule describes reversible recruitment of a TWEAK molecule from solution and formation of a 1:1 TWEAK-Fn14 complex. The forward and reverse rate constants associated with the reactions defined by this rule are k_{on} and k_{off}, respectively.

```
TWEAK(r,r,r) + Fn14(l) <-> TWEAK(r!1,r,r).Fn14(l!1) k_on,k_off   (1)
```

Rule 2: This rule describes a reversible intracomplex binding between TWEAK and Fn14. A TWEAK can be recruited by a receptor which is already in a complex with another receptor via the constitutive assembly. In such case, the recruited TWEAK may use one of its unoccupied sites to rapidly engage the other (unoccupied) receptor in the complex. The forward rate constant for this interaction is k_{o1}. The reverse rate constant k_{off} is the same as in Rule 1.

```
TWEAK(r).Fn14(l) <-> TWEAK(r!1).Fn14(l!1) k_o1,k_off        (2)
```

Rule 3: This rule describes binding (crosslinking) between a Fn14-bound TWEAK molecule and a free Fn14 molecule. It is distinct from Rule 2, which requires the two receptors to be already in a complex. Associated forward rate constant is k_c. The reverse rate constant k_{off} is the same as in Rule 1.

```
TWEAK(r!1,r).Fn14(l!1) + Fn14(l) <->
                    TWEAK(r!1,r!2).Fn14(l!1).Fn14(l!2) kc,k_off  (3)
```

Rule 4: This rule defines self-association between Fn14 molecules via their constitutive binding sites. Associated forward and reverse rate constant are k_c and k_{-c}, respectively. The forward rate constant is the same as that in Rule 3.

```
Fn14(r) + Fn14(r) <-> Fn14(r!1).Fn14(r!1) kc,k_c            (4)
```

Rule 5: This rule defines an intracomplex binding between two receptors. It allows the terminal Fn14 receptors of a linear trimer to bind and form a ring structure (see the last two steps of Figure A.2). This fast intracomplex binding is associated with forward rate constant k_{o2} and reverse rate constant k_{-c}. The reverse rate constant is the same as that in

Rule 4.

```
Fn14(l,r,r!1).Fn14(l,r!1,r!2).Fn14(l,r!2,r) <->
        Fn14(l,r!3,r!1).Fn14(l,r!1,r!2).Fn14(l,r!2,r!3) k_o2,k_c  (5)
```

1.2. ACTIVATION OF NF-κB AND OTHER EVENTS DOWNSTREAM OF FN14

We model most of these interactions, which follow Fn14 trimerization in the cell plasma membrane, based on [Tay *et al.*, 2010]. Fn14 receptor trimers, formed either via constitutive assembly or TWEAK-mediated crosslinking, activate a protein called IKK kinase (IKKK). The activated IKKK activates another protein called IKK. Activated IKK phosphorylats a protein called IκBα. In its unphosphorylated form, IκBα remains in complex with NFκB in the cell cytoplasm and inhibits nuclear translocation (activation) of NFκB. Phosphorylation of IκBα by IKK dissociates this protein from NF-κB. The phosphorylated and free IκBα then degrades rapidly while the free NFκB translocates to the nucleus. Upon nuclear entry, NF-κB mediates transcription of A20, IκBα and Fn14 genes and synthesizes respective mRNAs. The mRNAs are then translated to respective proteins. The newly synthesized Fn14 proteins are added to the existing pool in the cell plasma membrane.

Rule 6: This rule defines activation of IKKK by a TWEAK-crosslinked Fn14 trimer in the plasma membrane (see Figure 2 in main text of Paper I). We denote the inactive and active form of this protein as $\texttt{IKKK}_n$ and $\texttt{IKKK}_a$, respectively. The rate of activation is defined by the following function: $k_{IKKKactivation} = \frac{k_a.k_{A20}}{k_{A20}+A20}$ [Tay *et al.*, 2010]. In this rate expression, $A20$ represents cellular concentration of the A20 protein and k_{A20} represents a Hill function constant. This rate expression accounts for the inhibitory effect of A20 on

IKKK activation (see Figure 2 in main text).

```
IKKK_n + TWEAK(r!+,r!+,r!+).Fn14(l!+).Fn14(l!+).Fn14(l!+) ->
    IKKK_a + TWEAK(r!+,r!+,r!+).Fn14(l!+).Fn14(l!+).Fn14(l!+)
                                                k_IKKKactivation()   (6)
```

Rule 7: This rule describes activation of IKKK by a constitutively-assembled Fn14 trimer, which is devoid of TWEAK. We consider only the stable trimers, which have a ring structure, mediate this activation. The rate of activation is defined by the following function: $\delta \times k_{IKKKactivation} = \delta \times \frac{k_a . k_{A20}}{k_{A20}+A20}$, where $0 \leq \delta \leq 1$. This factor δ incorporates a possibility that the constitutively-formed Fn14 trimers are less effective in activating IKKK compared to the TWEAK-crosslinked Fn14 trimers.

```
IKKK_n + Fn14(l,r!+,r!+).Fn14(l,r!+,r!+).Fn14(l,r!+,r!+) ->
    IKKK_a + Fn14(l,r!+,r!+).Fn14(l,r!+,r!+).Fn14(l,r!+,r!+)
                                          k_IKKKactivation()*delta   (7)
```

Rule 8: This rule describes IKKK inactivation in a first-order process. Associated rate constant is defined as k_i.

```
IKKK_a -> IKKK_n k_i                                                 (8)
```

Rule 9: This rule describes activation of IKK by its kinase IKKK. We denote inactive and active IKK as $\mathtt{IKK}_n$ and $\mathtt{IKK}_a$, respectively. The rate of activation is defined by the following function: $k_{IKKactivation} = k_1 \cdot IKKK_a^2$.

```
IKK_n -> IKK_a k_IKKactivation()                                     (9)
```

Rule 10: This rule defines deactivation of IKK by A20. The rate is described by the following function: $k_{IKKintermetiation} = \frac{k_3}{k_2}(k_2 + A20)$. In this expression, $A20$ is the concentration of A20, and k_2 and k_3 are two constants.

```
IKK_a -> IKK_i k_IKKintermetiation()
```
(10)

Rules 11 and 12: These rules describe transition of IKK in between three different states $\mathtt{IKK}_i$, $\mathtt{IKK}_{ii}$, and $\mathtt{IKK}_n$. The same rate constant k_4 is associated with these transitions.

```
IKK_i -> IKK_ii k_4
```
(11)

```
IKK_ii -> IKK_n k_4
```
(12)

Rules 13, 14 and 15: These rules describe interaction of nuclear NF-κB with the gene promoters of three different proteins. These proteins are A20, IκBα and Fn14. Nuclear NF-κB is denoted as `NFkB(loc~n,bin)`. Here, `bin` denotes that NF-κB is not bound to IκBα whereas `bin!1` denotes NF-κB–IκBα binding as can be seen in the binding reactions, Rule 28 and 29. The genes corresponding to the three proteins in their free states are denoted as `GA20(st~0)`, `GIkBa(st~0)` and `GFn14(st~0)`, respectively. The NF-κB-bound state of these genes are denoted as `GA20(st~1)`, `GIkBa(st~1)` and `GFn14(st~1)`, respectively. All these rules are associated with the same rate constant q_1.

```
NFkB(loc~n,bin) + GA20(st~0) -> NFkB(loc~n,bin) + GA20(st~1) q_1
```
(13)

```
NFkB(loc~n,bin) + GIkBa(st~0) -> NFkB(loc~n,bin) + GIkBa(st~1) q_1
```
(14)

```
NFkB(loc~n,bin) + GFn14(st~0) -> NFkB(loc~n,bin) + GFn14(st~1) q_1f
```
(15)

Rules 16, 17 and 18: These rules describe deactivation of the above three genes by unphosphorylated nuclear IκBα with rate constant q_2. Nuclear IκBα is denoted as `IkBa(loc~n,pho~0,bin)`, where `loc~n` designates its location in nucleus, and `pho~0` designates its unphosphorylated state.

```
IkBa(loc~n,pho~0,bin) + GA20(st~1) ->
                        IkBa(loc~n,pho~0,bin) + GA20(st~0) q_2   (16)

IkBa(loc~n,pho~0,bin) + GIkBa(st~1) ->
                       IkBa(loc~n,pho~0,bin) + GIkBa(st~0) q_2   (17)

IkBa(loc~n,pho~0,bin) + GFn14(st~1) ->
                       IkBa(loc~n,pho~0,bin) + GFn14(st~0) q_2   (18)
```

Rules 19, 20 and 21: These three rules define transcription of the above three genes into corresponding mRNAs. The mRNA molecules are denoted as `A20_mRNA()`, `IkBa_mRNA()`, and `Fn14_mRNA()`, respectively. Because transcription is mediated by NF-κB in all three cases, we consider the same rate constant c_1.

```
GA20(st~1) -> GA20(st~1) + A20_mRNA() c_1          (19)

GIkBa(st~1) -> GIkBa(st~1) + IkBa_mRNA() c_1       (20)

GFn14(st~1) -> GFn14(st~1) + Fn14_mRNA() c_1       (21)
```

Rules 22, 23 and 24: These rules define degradation of the above three mRNA molecules. We consider the same rate constant c_3 for these rules.

```
A20_mRNA() -> 0 c_3                                    (22)
```

```
IkBa_mRNA() -> 0 c_3                                   (23)
```

```
Fn14_mRNA() -> 0 c_3                                   (24)
```

Rules 25, 26 and 27: These rules describe translation of above three mRNA molecules into corresponding proteins. We consider the same rate constant c_4 associated with these rules.

```
A20_mRNA() -> A20_mRNA() + A20() c_4                   (25)
```

```
IkBa_mRNA() -> IkBa_mRNA() + IkBa(loc~c,pho~0,bin) c_4 (26)
```

```
Fn14_mRNA() -> Fn14_mRNA() + Fn14(l,r,r) c_4f           (27)
```

Note that each of the above rule lumps together two different events: translation of a protein and then its transport from the nucleus to their respective compartments. Thus, the newly synthesized A20 and IκBα are assumed located in the cytoplasm and Fn14 is assumed located in the plasma membrane.

Rule 28: This rule defines interaction between cytoplasmic NF-κB and IκBα. These two molecules form a cytoplasmic NF-κB–IκBα complex. The binding is denoted by `bin!1`. The associated rate constant is a_1.

```
NFkB(loc~c,bin) + IkBa(loc~c,pho~0,bin) ->
                    NFkB(loc~c,bin!1).IkBa(loc~c,pho~0,bin!1) a_1  (28)
```

Rule 29: This rule defines interaction between nuclear NF-κB and IκBα. These two molecules form a nuclear NF-κB–IκBα complex. The reaction described by this rule is similar to that in Rule 28. However, the rate constant is scaled through multiplying a_1 by the cytoplasmic to nuclear volume ratio k_v. Thus, the associated rate constant is $k_{NF\kappa BI\kappa B} = a_1.k_v$.

```
NFkB(loc~n,bin) + IkBa(loc~n,pho~0,bin) ->
        NFkB(loc~n,bin!1).IkBa(loc~n,pho~0,bin!1) k_NFkBIkB
```
(29)

Rule 30: This rule describes IKK-mediated phosphorylation of cytoplasmic free IκBα with rate constant a_2.

```
IkBa(loc~c,pho~0,bin) + IKK_a -> IkBa(loc~c,pho~p,bin) + IKK_a a_2
```
(30)

Rule 31: This rule describes IKK mediated phosphorylation of IκBα within the cytoplasmic NF-κB–IκBα complex. Associated rate constant is a_3.

```
NFkB(loc~c,bin!1).IkBa(loc~c,pho~0,bin!1) + IKK_a ->
        NFkB(loc~c,bin!1).IkBa(loc~c,pho~p,bin!1) + IKK_a a_3
```
(31)

Rule 32: This rule describes degradation of A20 with rate constant c_5.

```
A20() -> 0 c_5
```
(32)

Rules 33 and 34: These two rules describe degradation of cytoplasmic phosphorylated and unphosphorylated IκBα, respectively. Associated rate constants are t_p and c_{5a} respectively.

```
IkBa(loc~c,pho~p,bin) -> 0 t_p
```
(33)

```
IkBa(loc~c,pho~0,bin) -> 0 c_5a
```
(34)

Rules 35 and 36: These two rules respectively describe ubiquitination (degradation) of phosphorylated and unphosphorylated IκBα within the NF-κB–IκBα complex. These reactions happen in a single step freeing NF-κB into the cytoplasm. The rate constants associated are t_p and c_{6a}, respectively.

```
NFkB(loc~c,bin!1).IkBa(loc~c,pho~p,bin!1) -> NFkB(loc~c,bin) t_p
```
(35)

```
NFkB(loc~c,bin!1).IkBa(loc~c,pho~0,bin!1) -> NFkB(loc~c,bin) c_6a
```
(36)

Rules 37: This rule describes degradation of membrane Fn14. Corresponding rate constant is d_f.

```
Fn14() -> 0 d_f
```
(37)

Rules 38 and 39: These two rules respectively describe the transport of free cytoplasmic NF-κB and unphosphorylated IκBα from the cytoplasm to the nucleus. Associated rate constants are i_1 and i_{1a}, respectively.

```
NFkB(loc~c,bin) -> NFkB(loc~n,bin) i_1
```
(38)

```
IkBa(loc~c,pho~0,bin) -> IkBa(loc~n,pho~0,bin) i_1a
```
(39)

Rules 40 and 41: These rules respectively describe the export of free and NF-κB-complexed IκBα from the nucleus to cytoplasm. Associated rate constants are e_{1a} and e_{2a}, respectively.

```
IkBa(loc~n,pho~0,bin) -> IkBa(loc~c,pho~0,bin) e_1a        (40)
```

```
NFkB(loc~n,bin!1).IkBa(loc~n,pho~0,bin!1) ->
                NFkB(loc~c,bin!1).IkBa(loc~c,pho~0,bin!1) e_2a  (41)
```

2. MOLECULES AND COPY NUMBERS

Table A.1 provides the list of basic protein molecules and their nominal copy numbers in the basal steady-state condition.

Table A.1. Molecules and their nominal copy numbers in cells.

Molecule	**Copy number**	**#/cell**	**Ref.**
Fn14	n_{fn14}	1.3×10^4	Fick *et al.* [2012]
NF-κB	n_{nfkb}	10^5	Tay *et al.* [2010]
IKK	n_{IKK}	10^5	Tay *et al.* [2010]
IKKK	n_{IKKK}	2×10^5	Tay *et al.* [2010]
IκBα gene	$n_{ikb\alpha}$	2	Tay *et al.* [2010]
A20 gene	n_{a20g}	2	Tay *et al.* [2010]
Fn14 gene	n_{fn14g}	2	Tay *et al.* [2010]

3. MODEL PARAMETERS

Table A.2 provides the list of parameters and their nominal values used in the model.

Table A.2. Model Parameters.

Description	**Parameter**	**Value**	**Ref.**
Rule 1: TWEAK (solution)-Fn14 (membrane) binding[1]	k_{on}	1.095×10^{-9} molecule^{-1}.s^{-1}	Fick *et al.* [2012]
Rule 1: TWEAK-Fn14 dissociation	k_{off}	0.58×10^{-3} s^{-1}	Fick *et al.* [2012]
Rule 2: Fn14 homotrimer ring-closure	k_{o1}	7.3×10^{3} s^{-1}	Khetan and Barua [2019]
Rule 3: Constitutive Fn14 association	k_c	1.095×10^{-7} molecule^{-1}.s^{-1}	Khetan and Barua [2019]
Rule 4: Constitutive Fn14 dissociation	k_{-c}	1.6 s^{-1}	Khetan and Barua [2019]
Rule 5: TWEAK-Fn14 trimer ring-closure	k_{o2}	6.85×10^{4} s^{-1}	Khetan and Barua [2019]

Continued on next page

[1]Fick et al. report k_{on} in molar units to be 3.3×10^6 M^{-1}·s^{-1} [Fick *et al.*, 2012]. To be consistent with the number units used in BioNetGen, k_{on} is converted to units of molecule^{-1}·s^{-1} using the equation $k_{on} = k_{on,molar}/(NA \cdot V)$, where NA is the Avogadro constant and V is the solution volume per cell. Cells were cultured at a density of 2×10^5 cells/mL making $V = 5 \times 10^{-9}$ L/cell. $\implies k_{on} = 3.3 \times 10^6/(6.022 \times 10^{23} \times 5 \times 10^{-9}) = 1.095 \times 10^{-9}$ molecule^{-1}.s^{-1}.

Table A.2. Model Parameters. (cont.)

Description	**Parameter**	**Value**	**Ref.**
Rule 6: IKKK activation by a TWEAK-crosslinked Fn14 trimer	$\frac{k_a . k_{A20}}{k_{A20}+A20}$		Tay *et al.* [2010]
	k_a	10^{-5} s^{-1}	Tay *et al.* [2010]
	k_{A20}	10^5	Tay *et al.* [2010]
Rule 7: IKKK activation by a constitutively assembled Fn14 trimer	$\delta \frac{k_a . k_{A20}}{k_{A20}+A20}$		Khetan and Barua [2019]
	δ	$2 \times 10^{-4} - 10^{-2}$	Khetan and Barua [2019]
Rule 8: IKKK inactivation	k_i	10^{-2} s^{-1}	Tay *et al.* [2010]
Rule 9: IKK activation	$k_1 \cdot IKKK_a^2$		Tay *et al.* [2010]
	k_1	6×10^{-10} s^{-1}	Tay *et al.* [2010]
Rule 10: IKK inactivation	$(k_2 + A20) \cdot \frac{k_3}{k_2}$		Tay *et al.* [2010]
	k_2	10^4	Tay *et al.* [2010]

Continued on next page

Table A.2. Model Parameters. (cont.)

Description	**Parameter**	**Value**	**Ref.**
	k_3	2×10^4 s^{-1}	Tay *et al.* [2010]
Rule 11, 12: IKK$_i$→IKK$_{ii}$ and IKK$_{ii}$→IKK$_n$	k_4	10^{-3} s^{-1}	Tay *et al.* [2010]
Rule 13, 14: NF-κB binding to A20 and IκBα gene promoters	q_1	4×10^{-7} s^{-1}	Tay *et al.* [2010]
Rule 15: NF-κB binding to Fn14 gene promoter	q_{1f}	1.2×10^{-6} s^{-1}	Khetan and Barua [2019]
Rule 16, 17, 18: IκBα induced NF-κB release from gene promoters	q_2	10^{-6} s^{-1}	Tay *et al.* [2010]
Rule 19, 20, 21: A20, IκBα, or Fn14 mRNA synthesis	c_1	10^{-1} s^{-1}	Tay *et al.* [2010]
Rule 22, 23, 24: A20, IκBα, or Fn14 mRNA degradation	c_3	7.5×10^{-4} s^{-1}	Tay *et al.* [2010]
Rule 25, 26: A20 or IκBα mRNA translation	c_4	0.5 s^{-1}	Tay *et al.* [2010]
Rule 27: Fn14 mRNA translation	c_{4f}	0.167 s^{-1}	Khetan and Barua [2019]

Continued on next page

Table A.2. Model Parameters. (cont.)

Description	**Parameter**	**Value**	**Ref.**
Rule 28, 29: NF-κB and IκBα association	a_1	5×10^{-7} s^{-1}	Tay *et al.* [2010]
Rule 30: Free IκBα phosphorylation	a_2	10^{-7} s^{-1}	Tay *et al.* [2010]
Rule 31: IκBα phosphorylation in NF-κB–IκBα complex	a_3	5×10^{-7} s^{-1}	Tay *et al.* [2010]
Rule 32: A20 protein degradation	c_5	5×10^{-4} s^{-1}	Tay *et al.* [2010]
Rule 33, 35: Phosphorylation-induced degradation of IκBα	t_p	10^{-2} s^{-1}	Tay *et al.* [2010]
Rule 34: Spontaneous degradation of free IκBα	c_{5a}	10^{-4} s^{-1}	Tay *et al.* [2010]
Rule 36: Spontaneous degradation of IκBα in NF-κB–IκBα complex	c_{6a}	2×10^{-5} s^{-1}	Tay *et al.* [2010]
Rule 37: Fn14 degradation	d_f	2.2×10^{-4} s^{-1}	Khetan and Barua [2019]
Rule 38: NF-κB nuclear import	i_1	10^{-2} s^{-1}	Tay *et al.* [2010]

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Table A.2. Model Parameters. (cont.)

Description	**Parameter**	**Value**	**Ref.**
Rule 39: IκBα nuclear import	i_{1a}	2×10^{-3} s^{-1}	Tay *et al.* [2010]
Rule 40: IκBα nuclear export	e_{1a}	5×10^{-3} s^{-1}	Tay *et al.* [2010]
Rule 41: NF-κB nuclear export	e_{2a}	5×10^{-2} s^{-1}	Tay *et al.* [2010]
Ratio of cytoplasmic to nuclear volumes	k_v	5	Tay *et al.* [2010]

APPENDIX B.

SUPPLEMENTARY FIGURES

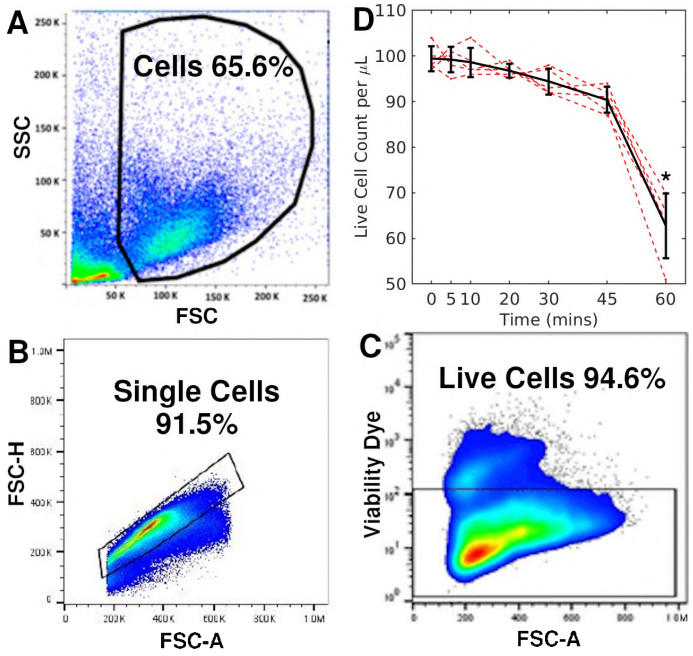

Figure B.1. Flow-cytometry sequential gating and cell counting. MDAMB-231 cells were grown on 24 well plates at a density of approximately 200,000 cells per well and treated with TWEAK 100 ng/mL for up tp 60 minutes. Cells were detached and re-suspended in 1 mL of PBS for flow-cytometry. Flow-cytometry data was sequentially gated in 3 steps as shown in panels A, B and C. (A) Removal of debris, (B) Removal of doublets, (C) Removal of dead cells stained with propidium iodide. (D) The remaining number of 'live cells' were counted using the manufacturer's cell counting protocol. The plot shows 5 replicates of >50,000 cells each (red) and the average live cell count at each time point (black).

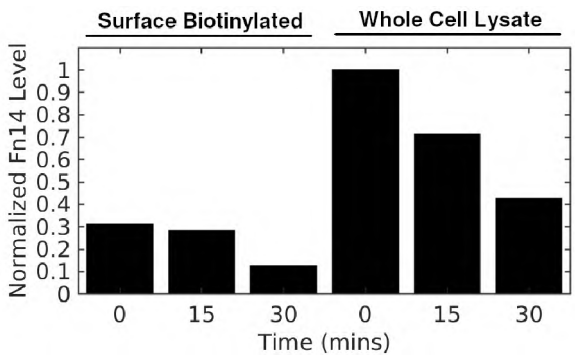

Figure B.2. Quantification of surface and cytoplasmic Fn14. Fn14 overexpressing cervical carcinoma HeLa cells were seeded at 37°C and surface proteins were biotinylated. Cells were then detached either immediately after biotinylation (0 min) or after 15 and 30 minutes of culturing. Whole cell lysates were prepared and either directly (Lysate) or after biotin pulldown using NeutrAvidin beads analyzed by Western blotting for the indicated proteins [Gurunathan *et al.*, 2014] (*the western blots can be found on Figure 2F of the publication*). The bar chart shows Fn14 blots quantified using ImageJ gel blot analyzer, normalized against EGFR levels and further normalized against whole cell lysate Fn14 level. The 0 min 'surface biotinylated' blot represents the surface Fn14 level while 0 min 'whole cell lysate' blot represents the total cell Fn14 level.

APPENDIX C.

SPATIAL EFFECTS IN SIGNALING PATHWAYS

A NOTE ON SPATIAL EFFECTS IN SIGNALING PATHWAYS

Spatial distribution of molecules can be of critical importance to cellular pathways. Signaling events initiated within the two dimensional plane of the membrane move through the three dimensional volume of the cytosol and propagate through multiple intracellular compartments. The simplest signaling model ignores transport limitations and defines each reaction step simply in terms of mass-action kinetics. This removes all partial differential equations and results in a purely ODE based model. On the other hand, the most complex model accounts for the diffusivity of each protein throughout the various sub-cellular compartments resulting in a large number of PDE's and ODE's. The model presented in this dissertation takes an intermediate approach where each cell is divided into three 'compartments' and protein transport rate is only considered when the protein moves from one compartment to the other (Figure N1). Protein-protein interactions within a compartment are assumed to be free of transport limitations.

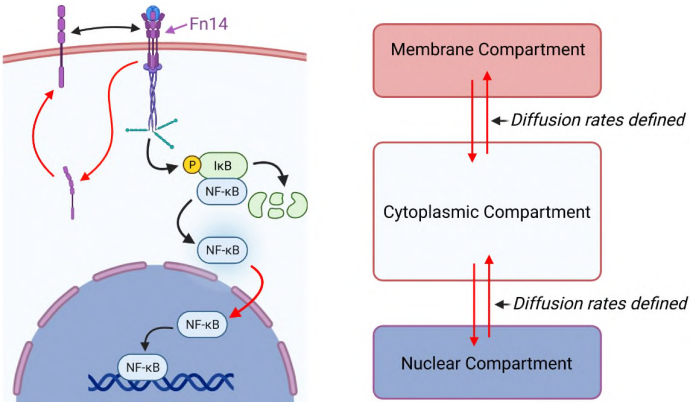

Figure C.1. A simplified pathway schematic is shown on the left. The cell is divided into three compartments: membrane, cytoplasm and nucleus. Signaling steps for which diffusion rates are defined are shown with red arrows. However, within each compartment, transport limitations are ignored.

The justification for this approach lies in the fact that transport of a protein across the plasma membrane or the nuclear envelope is in general one to two orders of magnitude slower than diffusion within a compartment [Cowan *et al.*, 2012; Timney *et al.*, 2016]. To demonstrate this, we may compare the approximate flux of the NFκB protein across the nuclear membrane with the flux of NFκB from the edge of the nucleus to the center of the nucleus. The flux across the nuclear membrane [Timney *et al.*, 2016] can be approximated as follows:

$$F_m = D_m(C_{cytoplasm} - C_{nucleus}) \tag{1}$$

Where, D_m is the diffusion coefficient for the movement of NFκB across the nuclear membrane and C represents the concentration of NFκB. D_m was calculated by Timney et al. [Timney *et al.*, 2016] for 50kDa proteins (like NFκB) to be 1.86×10^{-8}m/s. Assuming a cytoplasmic concentration of 50 μM (0.05 mol/m^3) and a nuclear concentration of 0 μM (fastest diffusion scenario), the flux is approximated to be:

$$F_m = 1.86 \times 10^{-8}(0.05 - 0) = 9.3 \times 10^{-10} mol/m^2 \tag{2}$$

Similarly, the flux of NFκB from the inside edge of the nucleus to the center of the nucleus is approximated as follows:

$$F_n = D_n \frac{dC_{nucleus}}{dx} \approx D_n \frac{\Delta C_{nucleus}}{\Delta x} = D_n \frac{C_{nucleus} - C_0}{r} \tag{3}$$

Where, D_n is the diffusivity of NFκB in the nucleoplasm, C_0 is the concentration of NFκB in the center of the nucleus and r is the radius of the nucleus. D_n for nuclear proteins was approximated [Kühn *et al.*, 2011] to be 1.72×10^{-11} m^2/s and the typical radius of mammalian nuclei can be assumed to be 3 μm. The flux of NFκB in the nucleus is therefore:

$$F_n = 1.72 \times 10^{-11} \frac{0.005 - 0}{3 \times 10^{-6}} = 3 \times 10^{-8} mol/m^2 \tag{4}$$

The flux of NFκB across the radius of the nucleus is therefore about 2 orders of magnitude higher than the flux across the nuclear membrane. Furthermore, the result assumes NFκB must move across the entire radius of the nucleus to reach the chromosomal matter; for transport over a comparable distance as the thickness of the nuclear membrane, the flux is over 5 orders of magnitude higher than the flux across the membrane. This calculation supports the assumption that transport effects on protein-protein interactions within a cellular compartment are significantly lower than the much higher transport limitations placed by membranes separating the various compartments.

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