Supplemental Materials

Encoding NF-κB temporal control in response to TNF: distinct roles for the negative feedback regulators IκBα and A20

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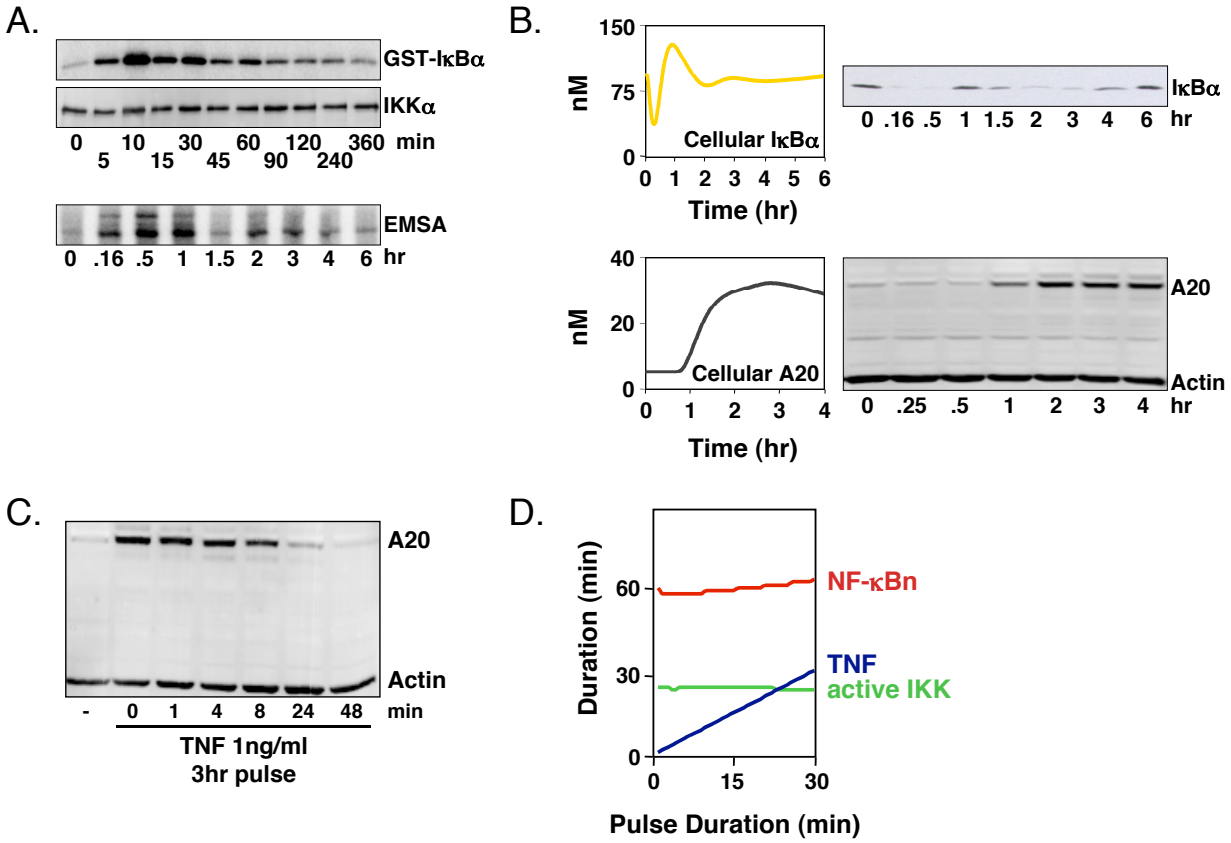


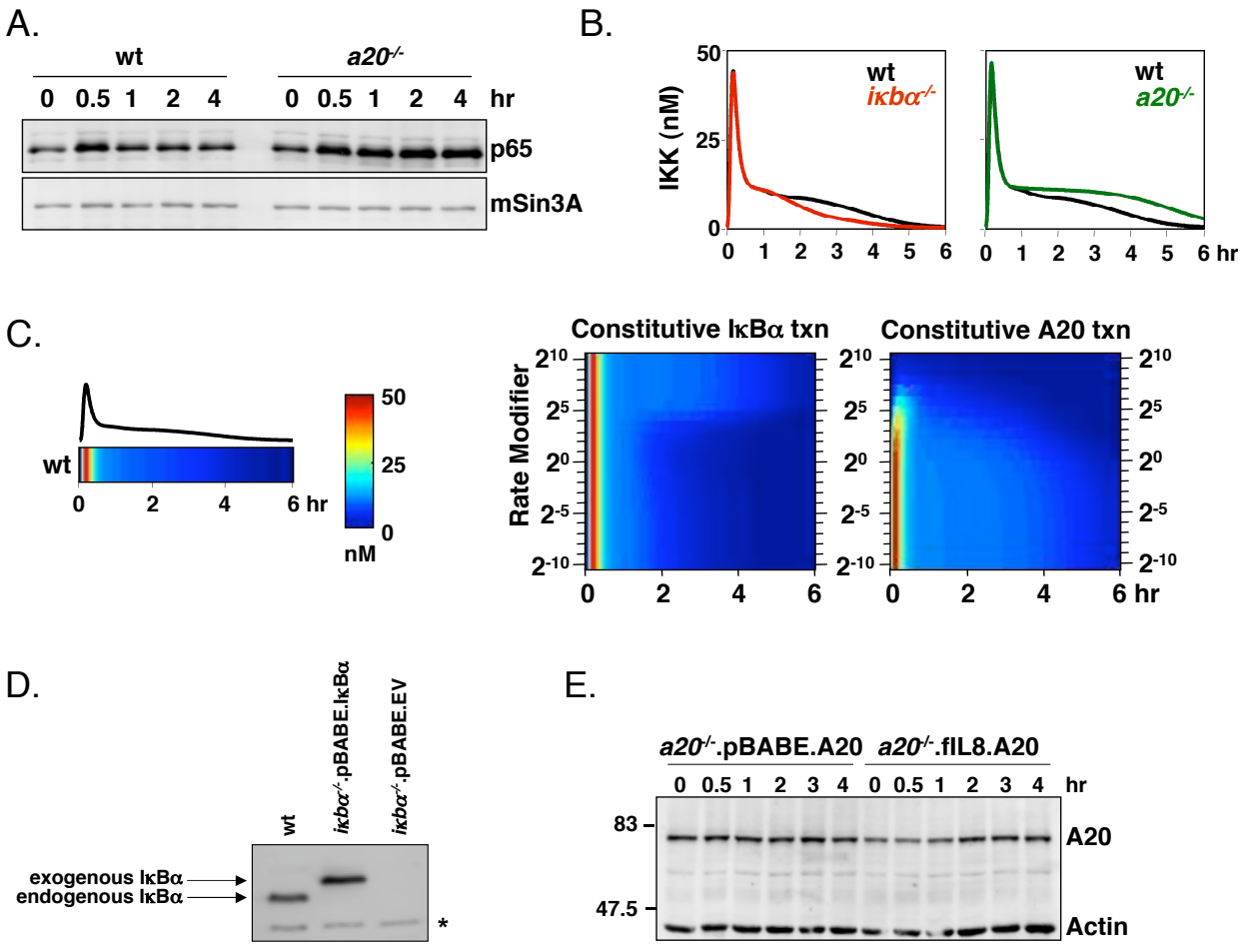
Supplemental Figure 1.

A. For model validation purposes, IKK (top) and NF-κB (bottom) activities were measured in response to 1ng/ml TNF in wild type cells, by *in vitro* kinase assay and electrophoretic mobility shift assay (EMSA), respectively.

B. IκBα and A20 protein levels were simulated in response to 1ng/ml TNF stimulation. Model outputs were validated by experimental measurements of both proteins via immunoblotting, where IκBα levels were measured in MEFs and A20 levels in HeLa cells. Antibodies for IκBα (1:5000) and A20 (1:100) were from Santa Cruz Biotechnology and Imgenex, respectively.

C. To estimate A20 protein halflife, HeLa cells were pretreated for 3hr with 1ng/ml TNF; this stimulation regimen was chosen because A20 mRNA is back to a basal expression level by this timepoint. The indicated timepoints were assayed for A20 and Actin protein expression via immunoblotting following stimulus removal. Basal A20 expression is denoted in the (-) lane. These data suggest that the halflife for A20 protein is approximately 8hr.

D. The model was simulated with pulses of increasing TNF duration (x-axis) and the resulting durations of IKK and NF-κB activities over a threshold of 20nM were calculated and plotted.



Supplemental Figure 2.

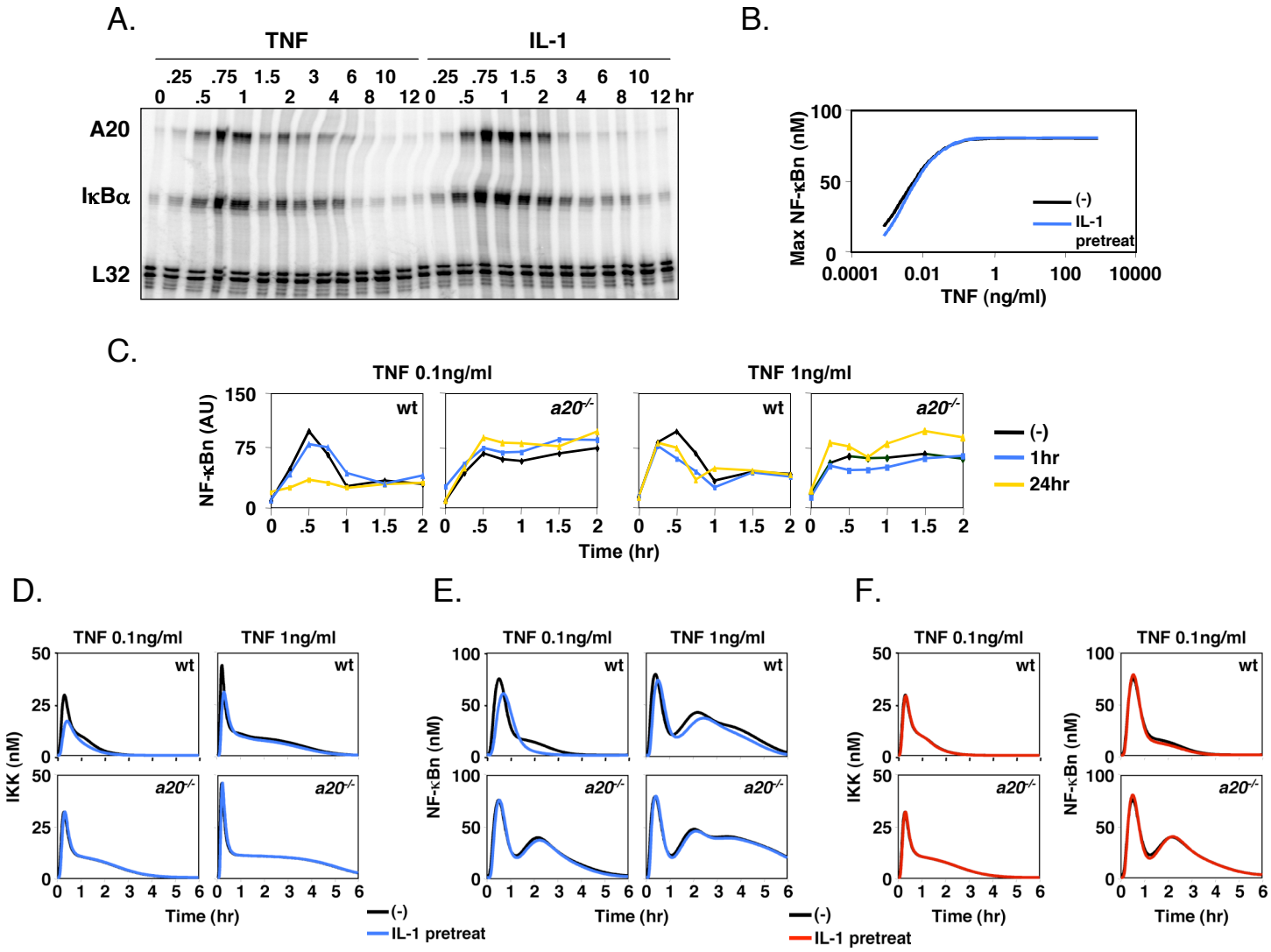
A. p65 nuclear localization was measured in nuclear extracts derived from wild type and *a20*$^{-/-}$ cells in response to 1ng/ml TNF. mSin3A was used as a nuclear localization control. p65 and mSin3A antibodies were from Santa Cruz Biotechnology, and used at a dilution of 1:1000 each.

B. IKK activation was simulated in wild type, *iκbα*$^{-/-}$ and *a20*$^{-/-}$ cells in response to TNF, as represented by line plots. IKK activation is normal in both knockouts; however, attenuation of late IKK activity is abrogated in the *a20*$^{-/-}$. The amplitude of late IKK activity has previously shown to be important for dictating the duration of NF-κB activity (Werner et al., 2005).

C. IKK activation in response to chronic TNF stimulation was modeled in wild type cells (left), which possess both constitutive and inducible IκBα/A20 transcription parameters. We then set either inducible IκBα (middle panel) or A20 (right panel) transcription rates to zero, while modulating the rate of constitutive transcription by 2^x (where $-10 \le x \le 10$), and plotted IKK activity over time as represented in a color heatmap, ranging from blue (0 nM) to red (50 nM).

D. Basal IκBα protein expression was measured via immunoblotting in cytoplasmic extracts in wild type and *iκbα*$^{-/-}$ MEFs retrovirally transduced with constitutively expressing human full-length IκBα or empty vector (EV). IκBα antibody was purchased from Santa Cruz Biotechnology and used at a dilution of 1:10,000. (*) denotes a non-specific band.

E. A20 protein expression was measured in whole cell RIPA lysates in cells retrovirally transduced with either constitutively (pBABE) or inducibly (fIL8) expressing A20 in response to 1ng/ml TNF.



Supplemental Figure 3.

A. IκBα and A20 mRNA levels were measured in wild type cells treated with either 1ng/ml TNF or IL-1 via RNase Protection Assay (RPA).

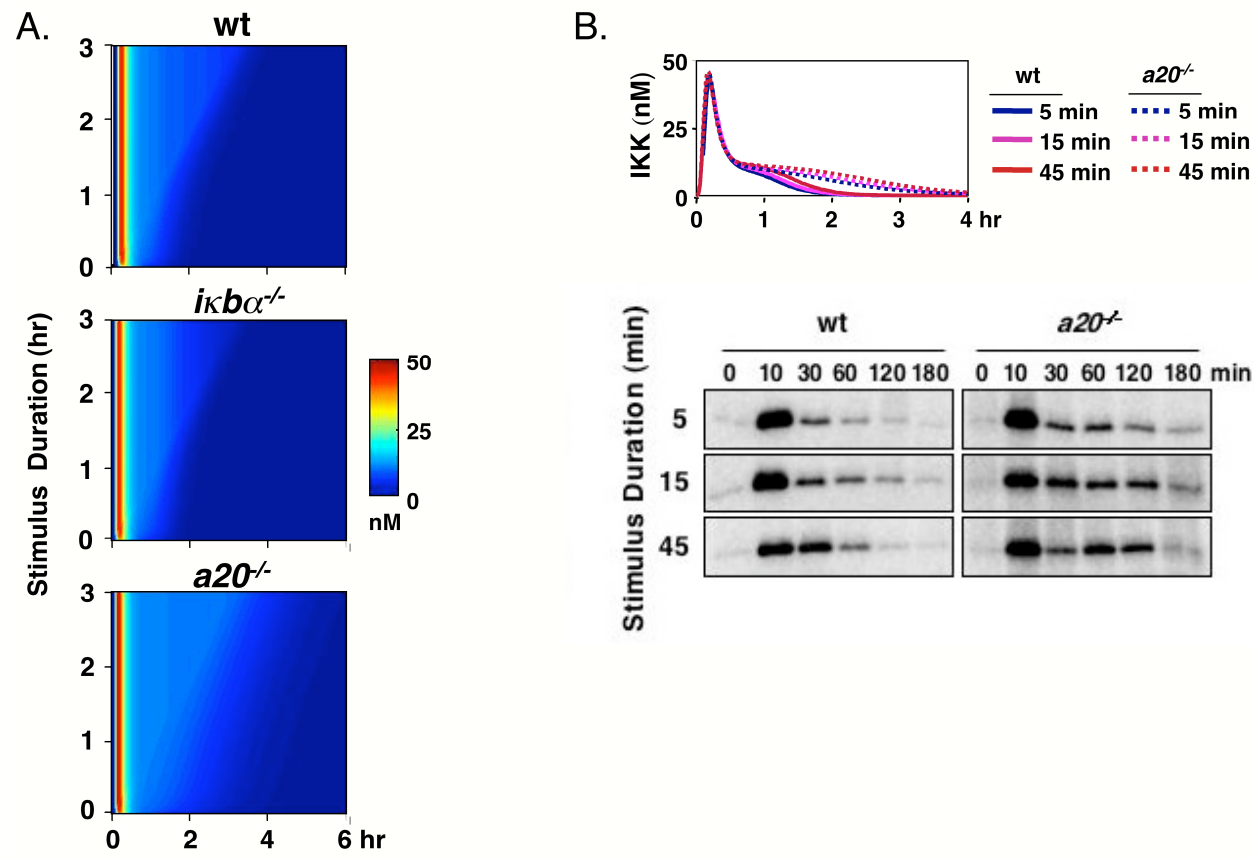
B. NF-κB activation in *a20*$^{-/-}$ cells was computationally simulated over time with a range of TNF doses ($10^{-3} \le x \le 10^{3}$). For each dose, maximal NF-κB activation (in nM) was plotted. The black line indicates TNF treatment alone, whereas the blue line represents a condition where cells were first pretreated with 1ng/ml IL-1, then allowed to "rest" (without stimulus) for 1hr prior to TNF stimulation. This result suggests that the IL-1 pretreatment condition has little to no effect on TNF-induced NF-κB activation in *a20*$^{-/-}$ cells.

C. NF-κB activation was measured in wild type and *a20*$^{-/-}$ cells via EMSA in response to either 0.1 or 1ng/ml TNF alone (black), or to a 1hr IL-1 pretreatment, followed by 1hr (yellow) or 24hr (blue) "rest" (no stimulation) and then subsequent TNF challenge, as shown in Figure 3D. The data was quantitated and graphed using ImageQuant software.

D. IKK activation was simulated in wild type and *a20*$^{-/-}$ cells in response to either 0.1 or 1ng/ml TNF alone (black), or to a 1hr IL-1 pretreatment, followed by 1hr "rest" (no stimulation) and then subsequent TNF challenge (blue).

E. NF-κB activation was simulated as in (D).

F. Computational simulations predict IKK (left) and nuclear NF-κB (right) activity in wild type and *a20*$^{-/-}$ cells that are pretreated with 1ng/ml IL-1 for 1hr, followed by 24hr "rest" (no stimulation), and then subsequent challenge with TNF (0.1ng/ml).



Supplemental Figure 4.

A. IKK was simulated as a function of time while varying the length of TNF pulse stimulation in wild type, *iκbα*$^{-/-}$ and *a20*$^{-/-}$ cells. Here we define a "temporal dose" as the duration of IKK activity in response to a specific TNF pulse length. The amplitude of IKK activity is represented on a color scale from 0 nM (blue) to 50 nM (red).

B. Computational simulations predict IKK activities (top) in response to 5, 15, or 45 min TNF pulses in wild type and *a20*$^{-/-}$ cells. IKK activity was then measured under the same stimulation conditions via IP-kinase assay (bottom).

Computational Modeling

A. Description of the Mathematical Model

The first version of this model (v1.0) was constructed to recapitulate TNF signaling to NF-κB via IKK activation (Hoffmann et al., 2002), but did not account for any reactions upstream of the NF-κB signaling module and contained only one negative feedback regulator, IκBα. Subsequent measurements of the *in vivo* degradation rates of free and NF-κB-bound IκB proteins led to model v1.1 (O'Dea et al., 2007), and inclusion of inducible IκBε negative feedback is described in model version 1.2 (Kearns et al., 2006). Subsequent models have examined NF-κB activation by multiple stimuli (v2.0) (Werner et al., 2005) and (v.3.0) (Basak et al., 2007).

A new model version (v4.0) was constructed for this study. The model is comprised of two connected modules (Supplemental Figure 5A). The first receives a dose of TNF ligand as an *input* and computes the activation of IKK as an *output*. The second module is based on a previous model (Werner et al., 2005) and uses the active IKK as an *input* and computes the activation of free nuclear NF-κB as an *output*. The complete new model contains 33 species (components) and 98 reactions governed by 110 parameters and describes the biochemical reactions involved in signal processing between TNF engagement of the TNF Receptors and nuclear NF-κB localization. The components of the model are depicted graphically in Supplemental Figure 5B and listed in Supplemental Table 1. The reactions and parameters are listed in Supplemental Table 2.

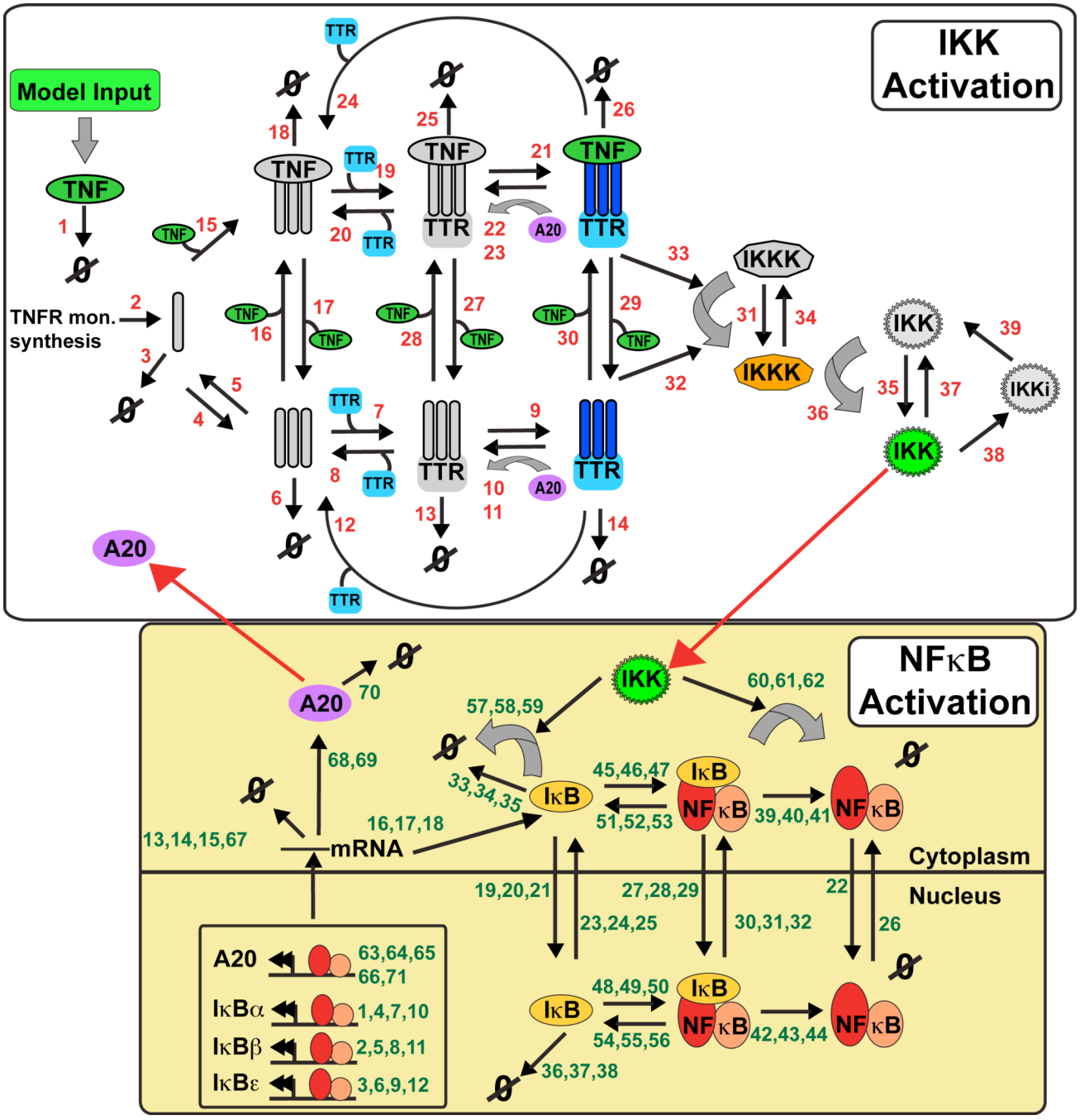
B. Computational Simulations

The ODEs were solved numerically using MATLAB version R2008a (The MathWorks, Inc.) with subroutine *ode15s*, a variable order, multi-step solver. Prior to stimulation, the system was allowed to equilibrate from starting conditions to a steady state, defined as showing no concentration changes greater than 1% over a period of 4000 minutes. Stimulus-induced perturbation from the equilibrium state was accomplished by introducing an extracellular concentration of TNF ligand or by direct modulation of IKK activity via a numerical input curve representing IL-1β stimulation (as in Werner et al., 2005).

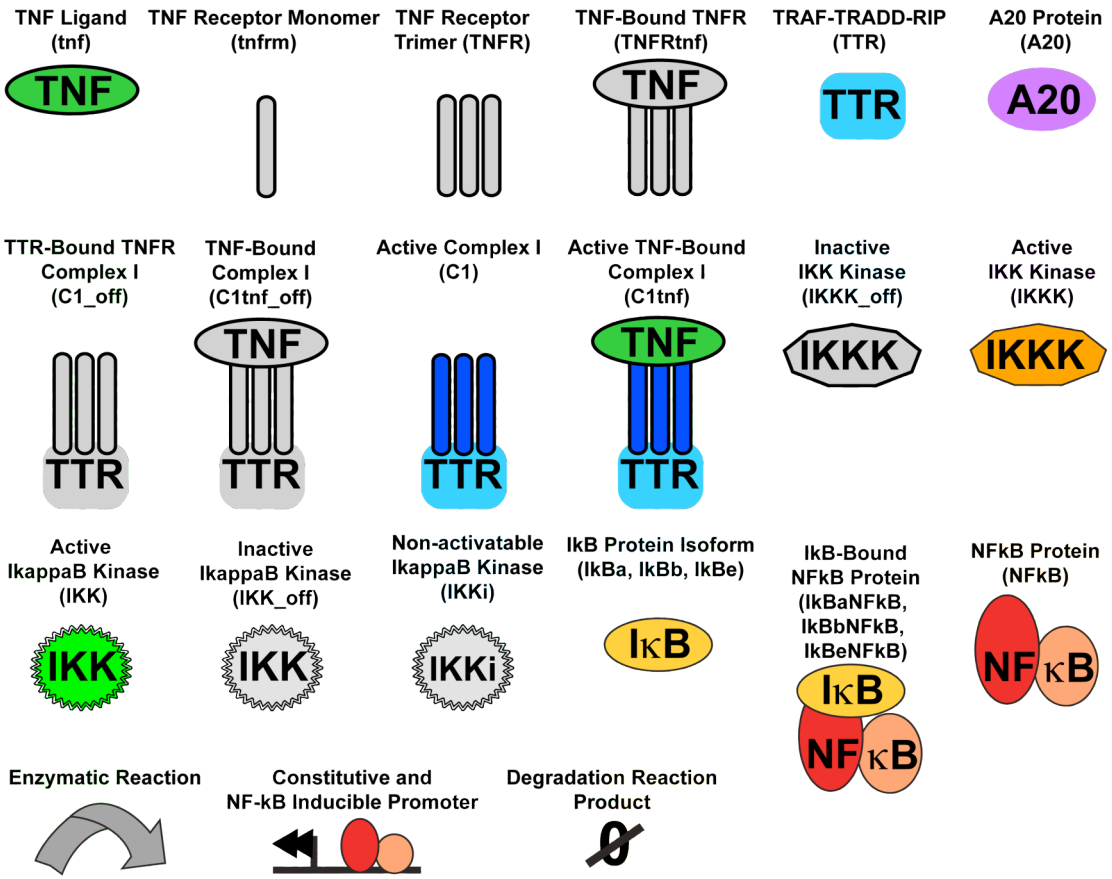
The model was simulated with multiple doses of TNF. We calculated that a 1ng/mL dose of TNF used in cell culture experiments was equivalent to 1.96e-4 μM in the model (the molecular weight of the TNF trimer is 45 kDa). Other concentrations were simulated in the model via multipliers of this number: ie: 0.1ng/mL is equivalent to 1.96e-5 μM.

The IL-1β numerical input curve was generated based upon IKK Kinase Assay measurements of IKK activity at time points following a 15 min pulse of IL-1β stimulation. A full curve was generated from these measurements in MATLAB using the built-in interpolation libraries with the Piecewise Cubic Hermite Interpolating Polynomial (PCHIP) method.

fraction IKK active	.01	.6	1	.65	.5	.36	.21	.16	.1	.01	.01
time (min)	0	5	10	15	20	25	30	45	50	60	120



Supplemental Figure 5A: Schematic Diagram of the Model Reaction Network
The model used in this study is comprised of two connected signaling modules that govern IKK activation and NF-κB activation, respectively. The input to the model is a concentration of TNF ligand. Numbers adjacent to reaction arrows denote individual model parameters and are listed in Supplemental Table 2.



Supplemental Figure 5B: Legend for the Schematic Network Diagram
There are 33 species (components) included in the model. Each is listed here with the molecular name first and the model nomenclature in parenthesis. For simplicity, there are only two components listed for IκB and the IκB:NF-κB complex. In the model there are separate components for each of the three IκB isoforms (IκBα, IκBβ, IκBε) in the cytoplasm and the nucleus.

	Model Species	Nomenclature	Initial μM	Location
1	IκBα	IkBa	0	Cytoplasm
2	IκBα	IkBan	0	Nucleus
3	IκBα-NF-κB	IkBaNFkB	0	Cytoplasm
4	IκBα-NF-κB	IkBaNFkBn	0	Nucleus
5	IκBα mRNA	IkBat	0	Cytoplasm
6	IκBβ	IkBb	0	Cytoplasm
7	IκBβ	IkBbn	0	Nucleus
8	IκBβ-NF-κB	IkBbNFkB	0	Cytoplasm
9	IκBβ-NF-κB	IkBbNFkBn	0	Nucleus
10	IκBβ mRNA	IkBbt	0	Cytoplasm
11	IκBε	IkBe	0	Cytoplasm
12	IκBε	IkBen	0	Nucleus
13	IκBε-NF-κB	IkBeNFkB	0	Cytoplasm
14	IκBε-NF-κB	IkBeNFkBn	0	Nucleus
15	IκBε mRNA	IkBet	0	Cytoplasm
16	A20	A20	0	Cytoplasm
17	A20 mRNA	A20t	0	Cytoplasm
18	NF-κB	NFkB	0	Cytoplasm
19	NF-κB	NFkBn	0.125	Nucleus
20	TNF	tnf	0	Extracellular
21	TNF Receptor Monomer	tnfrm	0	Cell Surface
22	TNF Receptor Trimer	TNFR	0	Cell Surface
23	TNF-Bound TNF Receptor Trimer	TNFRtnf	0	Cell Surface
24	TNFR Complex I (active)	C1	0	Cell Surface
25	TNFR Complex I (inactive)	C1_off	0	Cell Surface
26	TNF-Bound TNFR Complex I (active)	C1tnf	0	Cell Surface
27	TNF-Bound TNFR Complex I (inactive)	C1_tnf_off	0	Cell Surface
28	TRAF-TRADD-RIP	TTR	8.3e-4	Cytoplasm
29	TAK1 (active)	IKKK	0	Cytoplasm
30	TAK1 (inactive)	IKKK_off	0.1	Cytoplasm
31	IKK (active)	IKK	0	Cytoplasm
32	IKK (inactive)	IKK_off	0.1	Cytoplasm
33	IKK (auto-inactivated)	IKK_i	0	Cytoplasm

Supplementa1 Table 1
There are 33 species included in the model. Each is represented in the model with a unique name (nomenclature) and is given an initial concentration and cellular localization. The total amounts of NF-κB, TRAF-TRADD-RIP, TAK1 (IKKK) and IKK are conserved during the simulation (the sums of free, bound, active, and inactive forms in the cytoplasm and nucleus do not change).

Supplemental Table 2

NF-κB Activation Module					
IκB mRNA and Protein Synthesis Reactions					
#	**Reaction**	**Parameter Value**	**Category**	**Location**	**Source of Parameter Value**
1	=> IkBat (constitutive)	7 E-5 min^{-1}	RNA Synth.	-	Parameter value chosen to fit mRNA and protein expression profiles as measured by RNase Protection (RPA) and Western Blot assays.
2	=> IkBbt (constitutive)	1 E-5 min^{-1}	RNA Synth.	-	*Refer to #1.*
3	=> IkBet (constitutive)	1 E-6 min^{-1}	RNA Synth.	-	*Refer to #1.*
4 **7** **10**	=> IkBat (induced by NFkBn)	8 μM^{-2} min^{-1} Hill Coefficient: 3.0 Delay: 0 min	RNA Synth.	-	(Werner et al., 2005) (Werner et al., 2005) (Kearns et al., 2006) and unpublished results
5 **8** **11**	=> IkBbt (induced by NFkBn)	0.02 μM^{-2} min^{-1} Hill Coefficient: 3.0 Delay: 37 min	RNA Synth.	-	(Kearns et al., 2006) (Werner et al., 2005) (Kearns et al., 2006) and unpublished results
6 **9** **12**	=> IkBet (induced by NFkBn)	0.3 μM^{-2} min^{-1} Hill Coefficient: 3.0 Delay: 37 min	RNA Synth.	-	(Kearns et al., 2006) (Werner et al., 2005) (Kearns et al., 2006) and unpublished results
13	IkBat =>	0.035 min^{-1}	RNA Deg.	Cytoplasm	mRNA half-life measurements using actinomycin-D treatment of cells and RPA. (unpublished results)
14	IkBbt =>	3 E-3 min^{-1}	RNA Deg.	Cytoplasm	*Refer to #7.*
15	IkBet =>	4 E-3 min^{-1}	RNA Deg.	Cytoplasm	*Refer to #7.*
16	=> IkBa	0.25 min^{-1}	Prot. Synth.	Cytoplasm	(Hoffmann et al., 2002)
17	=> IkBb	0.25 min^{-1}	Prot. Synth.	Cytoplasm	(Hoffmann et al., 2002)
18	=> IkBe	0.25 min^{-1}	Prot. Synth.	Cytoplasm	(Hoffmann et al., 2002)
IκB and NFκB Cellular Localization Reactions					
19	IkBa => IkBan	0.09 min^{-1}	Import	-	(Werner et al., 2005)
20	IkBb => IkBbn	0.009 min^{-1}	Import	-	(Werner et al., 2005)
21	IkBe => IkBen	0.045 min^{-1}	Import	-	(Werner et al., 2005)
22	NFkB => NFkBn	5.4 min^{-1}	Import	-	(Werner et al., 2005)
23	IkBan => IkBa	0.012 min^{-1}	Export	-	(Werner et al., 2005)
24	IkBbn => IkBb	0.012 min^{-1}	Export	-	(Werner et al., 2005)
25	IkBen => IkBe	0.012 min^{-1}	Export	-	(Werner et al., 2005)
26	NFkBn => NFkB	0.0048 min^{-1}	Export	-	(Werner et al., 2005)
27	IkBaNFkB => IkBaNFkBn	0.276 min^{-1}	Import	-	(Werner et al., 2005)
28	IkBbNFkB => IkBbNFkBn	0.0276 min^{-1}	Import	-	(Werner et al., 2005)
29	IkBeNFkB => IkBeNFkBn	0.138 min^{-1}	Import	-	(Werner et al., 2005)
30	IkBaNFkBn => IkBaNFkB	0.828 min^{-1}	Export	-	(Werner et al., 2005)
31	IkBbNFkBn => IkBbNFkB	0.414 min^{-1}	Export	-	(Werner et al., 2005)
32	IkBeNFkBn => IkBeNFkB	0.414 min^{-1}	Export	-	(Werner et al., 2005)

IκB Protein Degradation Reactions					
33	IkBa =>	0.12 min^{-1}	Prot. Deg.	Cytoplasm	(O'Dea et al., 2007)
34	IkBb =>	0.18 min^{-1}	Prot. Deg.	Cytoplasm	(O'Dea et al., 2007)
35	IkBe =>	0.18 min^{-1}	Prot. Deg.	Cytoplasm	(O'Dea et al., 2007)
36	IkBan =>	0.12 min^{-1}	Prot. Deg.	Nucleus	(O'Dea et al., 2007)
37	IkBbn =>	0.18 min^{-1}	Prot. Deg.	Nucleus	(O'Dea et al., 2007)
38	IkBen =>	0.18 min^{-1}	Prot. Deg.	Nucleus	(O'Dea et al., 2007)
39	IkBaNFkB => NFkB	6E-5 min^{-1}	Prot. Deg.	Cytoplasm	(O'Dea et al., 2007)
40	IkBbNFkB => NFkB	6E-5 min^{-1}	Prot. Deg.	Cytoplasm	(O'Dea et al., 2007)
41	IkBeNFkB => NFkB	6E-5 min^{-1}	Prot. Deg.	Cytoplasm	(O'Dea et al., 2007)
42	IkBaNFkBn => NFkBn	6E-5 min^{-1}	Prot. Deg.	Nucleus	(O'Dea et al., 2007)
43	IkBbNFkBn => NFkBn	6E-5 min^{-1}	Prot. Deg.	Nucleus	(O'Dea et al., 2007)
44	IkBeNFkBn => NFkBn	6E-5 min^{-1}	Prot. Deg.	Nucleus	(O'Dea et al., 2007)
IκB:NFκB Association and Dissociation Reactions					
45	IkBa + NFkB => IkBaNFkB	30 μM^{-1} min^{-1}	Association	Cytoplasm	(Hoffmann et al., 2002)
46	IkBb + NFkB => IkBbNFkB	30 μM^{-1} min^{-1}	Association	Cytoplasm	(Hoffmann et al., 2002)
47	IkBe + NFkB => IkBeNFkB	30 μM^{-1} min^{-1}	Association	Cytoplasm	(Hoffmann et al., 2002)
48	IkBan + NFkBn => IkBaNFkBn	30 μM^{-1} min^{-1}	Association	Nucleus	(Hoffmann et al., 2002)
49	IkBbn + NFkBn => IkBbNFkBn	30 μM^{-1} min^{-1}	Association	Nucleus	(Hoffmann et al., 2002)
50	IkBen + NFkBn => IkBeNFkBn	30 μM^{-1} min^{-1}	Association	Nucleus	(Hoffmann et al., 2002)
51	IkBaNFkB => IkBa + NFkB	6E-5 min^{-1}	Dissociation	Cytoplasm	(Hoffmann et al., 2002)
52	IkBbNFkB => IkBb + NFkB	6E-5 min^{-1}	Dissociation	Cytoplasm	(Hoffmann et al., 2002)
53	IkBeNFkB => IkBe + NFkB	6E-5 min^{-1}	Dissociation	Cytoplasm	(Hoffmann et al., 2002)
54	IkBaNFkBn => IkBan + NFkBn	6E-5 min^{-1}	Dissociation	Nucleus	(Hoffmann et al., 2002)
55	IkBbNFkBn => IkBbn + NFkBn	6E-5 min^{-1}	Dissociation	Nucleus	(Hoffmann et al., 2002)
56	IkBeNFkBn => IkBen + NFkBn	6E-5 min^{-1}	Dissociation	Nucleus	(Hoffmann et al., 2002)
IKK-mediated IκB Degradation Reactions					
57	IkBa =>	0.36 min^{-1}	Prot. Deg.	Cytoplasm	(Mathes et al, 2008)
58	IkBb =>	0.12 min^{-1}	Prot. Deg.	Cytoplasm	(Mathes et al, 2008)
59	IkBe =>	0.18 min^{-1}	Prot. Deg.	Cytoplasm	(Mathes et al, 2008)
60	IkBaNFkB => NFkB	0.36 min^{-1}	Prot. Deg.	Cytoplasm	(Hoffmann et al., 2002)
61	IkBbNFkB => NFkB	0.12 min^{-1}	Prot. Deg.	Cytoplasm	(Hoffmann et al., 2002)
62	IkBeNFkB => NFkB	0.18 min^{-1}	Prot. Deg.	Cytoplasm	(Hoffmann et al., 2002)
A20 mRNA and Protein Synthesis and Degradation Reactions					
63	=> A20t (constitutive)	2 E-6 min^{-1}	RNA Synth.	-	*Refer to #1.*
64 **65** **66** **71**	=> A20t (induced by NFkBn)	0.4 μM^{-2} min^{-1} Hill Coefficient: 3.0 Delay: 0 min Shutdown: 120 min	RNA Synth.	-	- *Refer to #1.* - *Refer to #1.* - *Refer to #1.* - A20 inducible transcription, as measured by RPA, appears to halt abruptly 2hrs into TNF stimulation.
67	A20t =>	0.035 min^{-1}	RNA Deg.	Cytoplasm	*Refer to #1.*

68 **69**	=> A20	0.25 min^{-1} Delay Time: 30 min	Prot. Synth.	Cytoplasm	- Assumed to be equal to IκB translation rates. - Delay was added to account for time between A20 mRNA expression as measured by RPA and A20 protein expression as measured by Western Blot.
70	A20 =>	0.0029 min^{-1}	Prot. Deg.	Cytoplasm	Supplemental Figure 1
IKK Activation Module					
TNF-Independent Complex I Activity Reactions					
2	=> tnfrm	2 E-7 min^{-1}	Prot. Synth.	Cell Surface	Parameter value fit to recapitulate the measured steady-state amount of TNF receptor (Watanabe et al. 1988)
3	tnfrm =>	0.0058 min^{-1}	Prot. Deg.	Cell Surface	Measured in (Watanabe et al. 1988)
4	3 tnfrm => TNFR	1 E-5 μM^{-1} min^{-1}	Association	Cell Surface	Parameter value fit to account for minimal TNF receptor aggregation in the absence of ligand as observed in numerous published studies.
5	TNFR => 3 tnfrm	0.1 min^{-1}	Dissociation	Cell Surface	*Refer to #4.*
6	TNFR => (internalization)	0.0017 min^{-1}	Prot. Deg.	Cell Surface	Based upon results published in (Watanabe et al., 1988) showing that the temporal profile of TNF receptor following TNF stimulation.
7	TNFR + TTR => C1_off	100 μM^{-1} min^{-1}	Association	Cell Surface	Recruitment of TRAF2, TRADD, and RIP adaptors (TTR) to TNFR is required (but not sufficient) for signaling by the TNFR-containing signaling complex (C1). Little biophysical data is available for this reaction; recruitment appears to be simultaneous (Schneider-Brachert et al., 2004). The parameter value represents a compound mechanistic rate constant. It was fit to enable quick activation of downstream IKK activity within the first minutes of stimulation and repression upon removal of TNF ligand in pulse stimulations.
8	C1_off => TNFR + TTR	0.75 min^{-1}	Dissociation	Cell Surface	*Refer to #7.*
9	C1_off => C1	30 min^{-1}	Activation	Cell Surface	The molecular complex containing TNFR, TRAF2, TRADD and RIP undergoes an activation step that involves K63-ubiqutination of RIP. Little biophysical data is available for this step, but parameter fitting was constrained by the fast activation profile of IKK.
10	C1 => C1_off	2.0 min^{-1}	Deactivation	Cell Surface	*Refer to #9.*
11	C1 => C1_off (A20 mediated)	1000 μM^{-1} min^{-1}	Deactivation	Cell Surface	A20 is known to repress the activity of Complex I. It is a protease of K63-linked ubiquitin chains that deubiquitinates RIP (Wertz et al., 2004). Little biophysical data is available for this step, but parameter fitting was constrained by the IKK activity profiles measured in *wild type* and *a20*$^{-/-}$ cells.
12	C1 => TNFR + TTR	0.75 min^{-1}	Dissociation	Cell Surface	*Assumed to be equal to #8.*
13	C1_off => (internalization)	0.0017 min^{-1}	Prot. Deg.	Cell Surface	*Assumed to be equal to #6.*
14	C1 => (internalization)	0.0017 min^{-1}	Prot. deg.	Cell Surface	*Assumed to be equal to #6.*

TNF-Dependent Complex I Activity Reactions					
1	tnf =>	0.0154 min^{-1}	Prot. deg.	Extracellular	The half-life of recombinant TNF ligand in cell culture medium was measured by its manufacturer, Roche Diagnostics, to be 45-minutes.
15	tnf + 3 tnfrm => TNFRtnf	1100 μM^{-1} min^{-1}	Association	Cell Surface	Measured in (Grell et al., 1998).
16	tnf + TNFR => TNFRtnf	1100 μM^{-1} min^{-1}	Association	Cell Surface	*Assumed to be equal to #15.*
17	TNFRtnf => TNFR + tnf	0.021 min^{-1}	Dissociation	Cell Surface	Measured in (Grell et al., 1998).
18	TNFRtnf => (internalization				*Assumed to be equal to #6.*
19	TNFRtnf + TTR => C1tnf_off	100 μM^{-1} min^{-1}	Association	Cell Surface	*Assumed to be equal to #7.* TNF binding to the extra-cellular domain of TNFR monomers speeds up trimerization and stabilizes the trimer, but recruitment of the TTR complex to trimerized TNF receptor are assumed to proceed with the same kinetics regardless of the presence of TNF ligand.
20	C1tnf_off => TNFRtnf + TTR	0.75 min^{-1}	Dissociation	Cell Surface	*Refer to #19. Assumed to be equal to #8.*
21	C1tnf_off => C1tnf	30 min^{-1}	Activation	Cell Surface	*Refer to #19. Assumed to be equal to #9.*
22	C1tnf => C1tnf_off	2.0 min^{-1}	Deactivation	Cell Surface	*Refer to #19. Assumed to be equal to #10.*
23	C1tnf => C1tnf_off (A20 mediated)	1000 μM^{-1} min^{-1}	Deactivation	Cell Surface	*Refer to #19. Assumed to be equal to #11.*
24	C1tnf => TNFRtnf + TTR	0.75 min^{-1}	Dissociation	Cell Surface	*Refer to #19. Assumed to be equal to #8.*
25	C1tnf_off => (internalization)	0.0017 min^{-1}	Prot. deg.	Cell Surface	*Refer to #19. Assumed to be equal to #6.*
26	C1tnf => (internalization)	0.0017 min^{-1}	Prot. deg.	Cell Surface	Refer to #19. Assumed to be equal to #6.
27	C1tnf_off => C1_off + tnf	0.021 min^{-1}	Dissociation	Cell Surface	*Assumed to be equal to #17.*
28	C1_off + tnf=> C1tnf_off	1100 μM^{-1} min^{-1}	Association	Cell Surface	*Assumed to be equal to #15.*
29	C1tnf => C1 + tnf	0.021 min^{-1}	Dissociation	Cell Surface	*Assumed to be equal to #17.*
30	C1 + tnf => C1tnf	1100 μM^{-1} min^{-1}	Association	Cell Surface	*Assumed to be equal to #15.*
IKKK (TAB1/2-TAK1 complex) Activity Reactions					
31	IKKK_off => IKKK (constitutive)	5 E-7 min^{-1}	Activation	Cytoplasm	Parameter value fit to account for low IKK activity in the absence of ligand as measured by IKK Kinase Assay (O'Dea et al., 2007).
32	IKKK_off => IKKK (C1 mediated)	500 μM^{-1} min^{-1}	Activation	Cytoplasm	*Refer to #7.*
33	IKKK_off => IKKK (C1tnf mediated)	500 μM^{-1} min^{-1}	Activation	Cytoplasm	*Refer to #19. Assumed to be equal to #32.*
34	IKKK => IKKK_off (constitutive)	0.25 min^{-1}	Deactivation	Cytoplasm	The constitutive inactivation rate of this complex was fit to ensure low basal activity and efficient repression following TNF pulse stimulation.
IKK Activity Reactions					
35	IKK_off => IKK	5 E-5 min^{-1}	Activation	Cytoplasm	*Refer to #31*
36	IKK_off => IKK (IKKK mediated)	520 μM^{-1} min^{-1}	Activation	Cytoplasm	*Refer to #7.*
37	IKK => IKK_off	0.02 min^{-1}	Deactivation	Cytoplasm	*Refer to #34.*

38	IKK => IKK_i (self-inactivation)	0.15 min^{-1}	Deactivation	Cytoplasm	IKK is thought to down-regulate its own activity via auto-phosphorylation of C-terminal serine residues (Delhase et al., 1999). This mechanism was not shown to cause IKK protein degradation and is distinct from inactivating IKK *via* constitutive phosphatase activity (*Refer to* #94). The parameter value was fit to temporal profiles of IKK activity in response to TNF stimulation (Werner et al., 2005).
39	IKK_i => IKK_off	0.02 min^{-1}	Deactivation	Cytoplasm	C-terminally phosphorylated IKK is assumed to be subject to constitutive phosphatase activity. *Refer to #38*.
x	IL1_IKK Activity	Numerical Input Curve	-	-	Stimulation by IL1 is enabled through a numerical input curve (as first used in (Werner et al., 2005)) that specifies time-dependent activation kinetics of a pool of IL1-responsive IKK.

C. Parameter Fitting and Sensitivity Analysis

Most model parameter values were derived from the literature or measured / tightly constrained by biochemical or biophysical techniques. The values of remaining model parameters were selected such that the output of the model recapitulated observed results. This process is commonly referred to as parameter fitting and is essential when constructing a model representing a large reaction network.

Experiments yielded steady state and stimulation time course data that function to constrain the parameter fitting. The constraints were defined with broad ranges so as to minimize the possibility of arbitrarily biasing the results and were expected to be met in response to pulse or chronic stimulation with 1ng/mL TNF (Supplemental Table 3, Supplemental Fig 6).

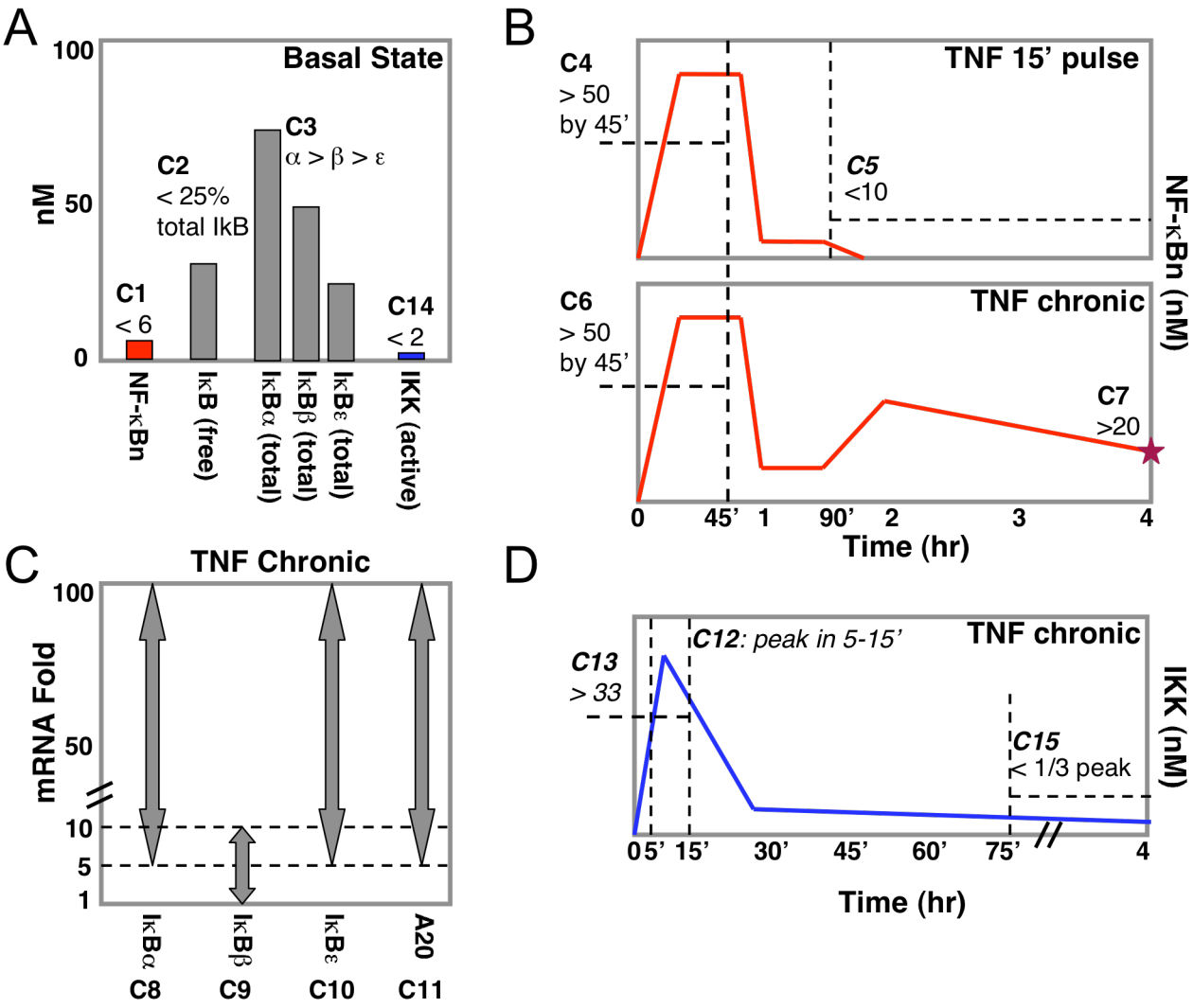
Supplemental Table 3: Model Constraints

	Description	**Source**
C1	Basal NF-κB activity is less than 5% of the total NF-κB	EMSA
C2	Basal free IκB protein is less than 25% of total IκB	(O'Dea et al., 2007)
C3	Basal total cellular protein of IκBα > IκBβ > IκBε	(O'Dea et al., 2007)
C4	15' TNF Pulse: NF-κB activity is > 50nM within 45 min	(Cheong et al., 2006)
C5	15' TNF Pulse: NF-κB activity is < 10nM by 90 min	(Cheong et al., 2006)
C6	TNF Chronic: NF-κB activity is > 50nM within 45 min	(Cheong et al., 2006)
C7	TNF Chronic: NF-κB activity is > 20nM at 240 min	(Cheong et al., 2006)
C8	TNF Chronic: IκBα mRNA induction fold is 5 to 100 fold	This study and (Kearns et al., 2006)
C9	TNF Chronic: IκBβ mRNA induction is 1 to 10 fold	(Kearns et al., 2006)
C10	TNF Chronic: IκBε mRNA induction is 5 to 100 fold	(Kearns et al., 2006)
C11	TNF Chronic: A20 mRNA induction is 5 to 100 fold	This study
C12	TNF Chronic: IKK activity peaks between 5 and 15 min	(Werner et al., 2005)
C13	TNF Chronic: peak IKK activity is above 33% of the total IKK	(Werner et al., 2005)
C14	Basal IKK activity is below 2% of the total IKK pool	(Werner et al., 2005)
C15	TNF Chronic: IKK activity (>75min) is < 1/3 of the peak value	(Werner et al., 2005)

Examining the values listed in Supplemental Table 2, we identified 15 parameters or groups of related parameters in each of the two signaling modules that were fit (Supplemental Figure 7A and 7B). We then determined for each of these parameters what ranges of their values would satisfy the established list of constraints. To do this, the model was simulated repeatedly with rate multipliers between 1/100 and 100x for each parameter. We calculated the highest and lowest multiplier values that when run in the model still satisfy all constraints (Supplemental Figure 7C and 7D).

We found that the majority of these parameters can be given values within a 2-5 fold range. This finding shows that the network allows for some degree of flexibility in parameter values while maintaining its function.

Four parameters can be given values over several orders of magnitude. These include the rates of inducible IκBβ expression, TNF-independent TNFR trimerization, constitutive IKKK activation, and constitutive IKK activation. These findings are not surprising as all four of these processes are kinetically 'slow' and the latter three are counteracted by fast reverse reactions.



Supplemental Figure 6: Diagrams of constraints used to constrain the model
(A) Maximum basal concentrations of specific model species
(B): Characteristics of NF-κBn curves in response to 15 min 1ng/mL TNF pulse or TNF chronic stimulation
(C): Allowable ranges for IκB and A20 mRNA induction folds in response to 1ng/mL TNF chronic stimulation
(D): Characteristics of the IKK activity curve in response to 1ng/mL TNF chronic stimulation.

A

NF-κB Activation Module

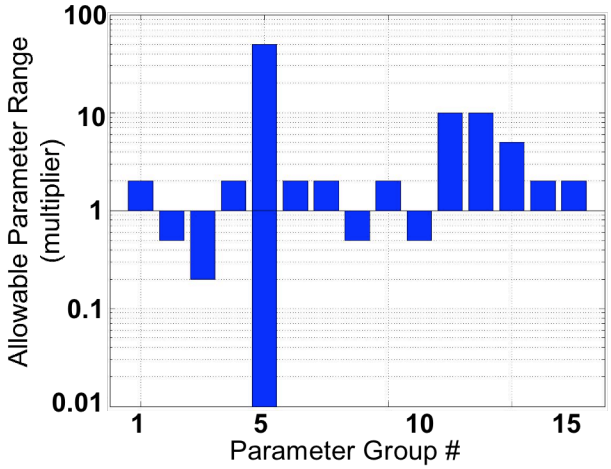
#	Parameter	Function
1	1	IκBα const. txn
2	2	IκBβ const. txn
3	3	IκBε const. txn
4	4	IκBα inducible txn
5	5	IκBβ inducible txn
6	6	IκBε inducible txn
7	16	IκBα translation
8	17	IκBβ translation
9	18	IκBε translation
10	57,60	IKK-IκBα Deg.
11	58,61	IKK-IκBβ Deg.
12	59,62	IKK-IκBε Deg.
13	63	A20 const. txn
14	64	A20 inducible txn
15	68	A20 translation

B

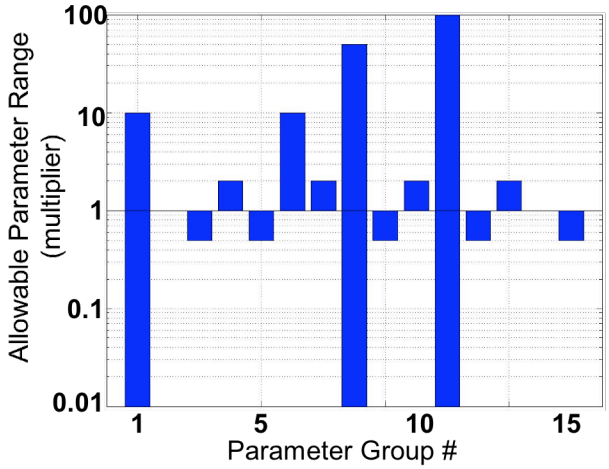
IKK Activation Module

#	Parameter	Function
1	4	TNFR trimerization
2	5	TNFR dissociation
3	7,19	TTR recruitment
4	8,12,20,24	TTR dissociation
5	9,21	ComplexI activation
6	10,22	ComplexI inactivation
7	11,23	A20 Complex1 inactivation
8	31	Const. IKKK activation
9	32,33	C1-mediated IKKK activation
10	34	Const. IKKK inactivation
11	35	Const. IKK activation
12	36	IKKK-mediated IKK activation
13	37	Const. IKK inactivation
14	38	IKK to IKKi conversion
15	39	IKKi to IKKoff conversion

C



D



Supplemental Figure 7: Determining the range of parameter values that satisfy the constraints

(A and B) There are 15 parameters or sets of related parameters in each signaling module that required parameter fitting to select their values.

(C and D) For each of these, the model was simulated repeatedly with that rate value(s) multiplied by 0.01, 0.02, 0.025, 0.1, 0.2, 0.5, 1, 2, 5, 10, 25, 50, and 100x. Following each simulation, the model was compared against the list of 13 established constraints. Plotted are the maximum and minimum multipliers for each rate constant group that still satisfies all constraints. The absence of a bar, such as with Groups 2 and 14 in the IKK module, means

D. Testing the Robustness of Conclusions

To determine whether the predictions of the model hold true over these ranges of parameter values that were defined by the experimental constraints, all model simulations relevant for the conclusions were repeated with the ranges of allowable values (Supplemental Figure 8). We identified 4 primary conclusions in our study (as described in the Abstract and Results/Discussion section).

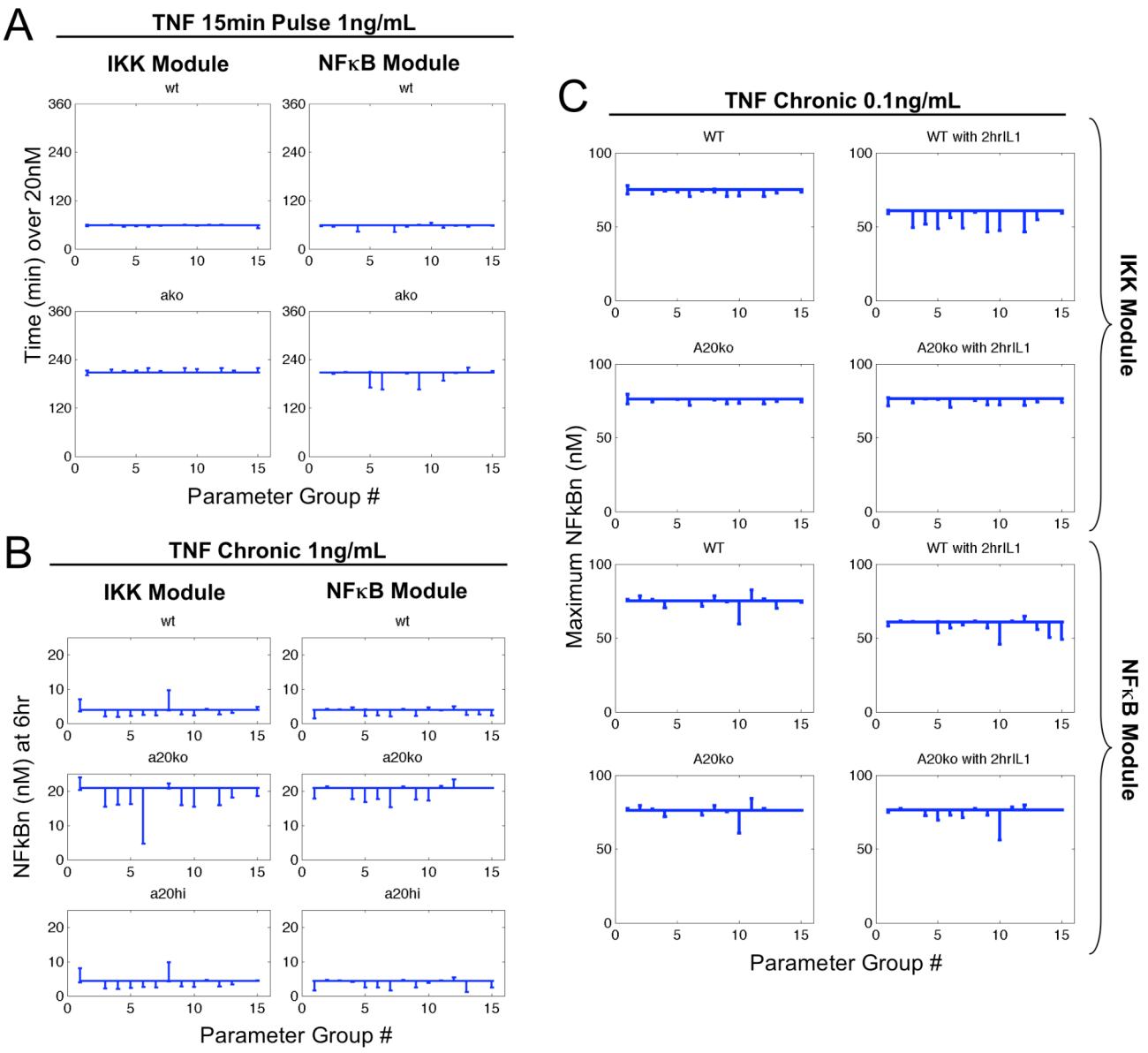
(1) *IκBα controls the duration of the first phase of NF-κB activity in response to a pulse of TNF stimulation*. NF-κB activity should last ~1hr in wild-type cells and be prolonged to ~3hr in IκBα-deficient cells (Supplemental Figure 8A).

(2) *A20 controls the amplitude of the second phase of NF-κB activity in response to chronic TNF stimulation* (Supplemental Figure 8B compare top and middle row).

(3) *Constitutive transcription of A20 is sufficient for A20's function in controlling late phase of NF-κB activity*. NF-κB-mediated inducible synthesis is not required for this behavior and can be replaced with elevated constitutive transcription (~4x) (Supplemental Figure 8B bottom row).

(4) *Pretreatment with IL-1β followed by treatment with TNF will result in lowered NF-κB activity in wild-type but not in A20-deficient cells*, and this effect is relived by 24 hrs (Supplemental Figure 8C)

We found that the model retains these conclusions when simulated with the parameter value ranges, with only small fluctuations observed. The exception is the rate constant governing constitutive Complex I deactivation (IKK Module Parameter Group #6), the same function performed by A20. This confirms that A20 represents a tunable Complex I deactivation mechanism. Together, this analysis leads us to believe that the model predictions are not impacted by the choice of parameter values.



Supplemental Figure 8: The model predictions are robust to the parameter range
The plots show how a metric that encapsulates each conclusion is affected by simulating the model with the calculated maximum and minimum values for each of the 15 parameters fit in the NF-κB and IKK activation modules. The horizontal line denotes a model with all parameters at 1x values. Error bars denote the deviation from 1x when using the range for each parameter.
(A) Wild-type and A20-/- model systems were stimulated with a 15 min TNF pulse and the duration of the NF-κB activity over a 20nM threshold was calculated.
(B) Wild-type, A20-/-, and A20 4x constitutive expressing (no inducible expression) systems were chronically stimulated with TNF and the value of NF-κB activity at 6hrs was calculated.
(C) Naïve and IL-1 pretreated wild-type and A20-/- systems were chronically stimulated with TNF and the maximum (peak) value of NF-κB activity in the first hour was calculated.

E. Supplemental Table 4-Summary of Simulations

Figure	Condition	Modeling Notes
1B	Wild type *Chronic TNF Stimulation*	Parameter values were set to those shown in the Supplemental parameter table. Following an equilibrium phase, TNF was added to the system (1.96e-4 μM, ~1ng/mL TNF) and the simulation was continued.
1C	Wild type *1, 2, 5, 15 minute TNF pulses*	Simulations were run with four TNF pulses (1ng/ml). TNF was removed from the system at the indicated pulse length times. Nuclear NF-κB and active IKK were plotted for each pulse.
2A	1. Wild type vs. $i\kappa b\alpha^{-/-}$ 2. Wild type vs. $a20^{-/-}$ *Chronic TNF Stimulation (1ng/ml)*	The $i\kappa b\alpha^{-/-}$ system was simulated after setting the initial values of IκBα-containing species and IκBα synthesis parameters to zero. The $a20^{-/-}$ system was simulated after setting the A20 synthesis parameters to zero.
2B **S2C**	Constitutive transcription multipliers for IκBα and A20. *Chronic TNF Stimulation (1ng/ml)*	For both IκBα and A20, simulations were run with 21 multipliers to constitutive transcription (2^{-10} ... 2^{0} ... 2^{10}; with 2^{0} (1x) being wild type). Results were plotted on a 3-dimensional plot—Time vs. Multiplier value using a color heat map to show nuclear NF-κB activity. (Similar plots are shown in Supplemental Figure 2C for IKK activity.)
2C	Wild type vs. IκBα constitutive transcription vs. IκBα inducible transcription. *15 minute TNF pulse (1ng/ml)*	The model was run three times with the following conditions: 1. Wild type 2. IκBα inducible synthesis parameter set to zero. 3. IκBα constitutive synthesis parameter set to zero.
2D	Wild type vs. A20 constitutive transcription vs. A20 inducible transcription. *Chronic TNF Stimulation (1ng/ml)*	The model was run three times with the following conditions: 1. Wild type 2. A20 inducible synthesis parameter set to zero, and the constitutive rate was set to 4x higher than wild type cells, as measured experimentally via qPCR 3. A20 constitutive synthesis parameter set to zero.
3C **S3D** **S3E** **S3F**	IL-1 pretreatment in wild type vs. $a20^{-/-}$. *IL-1 Pretreatment* *Chronic TNF Stimulation*	Wild type and $a20^{-/-}$ systems without IL-1 pretreatment were simulated as described in Figure 2A. IL-1 pretreatment was accomplished by incorporating a numerical IKK activity curve, as described previously (Werner 2005), that describes a 60-minute pulse of IL-1 stimulation. During the pretreatment phase the

		parameters governing the conversion amongst IKK forms (IKK, IKK_off and IKKi) were set to zero and the amount of active IKK was determined by the numerical curve. Following pretreatment, these parameters were restored and a range of TNF doses ($10^{-3} \leq x \leq 10^{3}$ ng/ml) were added. Maximal NF-κB activity (nM) was plotted as a function of TNF dose in both naïve and IL-1 pretreated cells. Specific TNF doses (0.1 and 1ng/ml) were modeled in naïve and IL-1 pretreated cells, and the results for IKK and NF-κB activities are shown in Supplemental Figures 3D and 3E, respectively. IKK and NF-κB activities were also simulated in a condition where 24hr rest was allowed (rather than 1hr) after IL-1 pretreatment; the resulting output is presented in Supplemental Figure 3F. Experimental results for NF-κB activity are shown in Figure 3D and are quantitated in Supplemental Figure 3C.
4A **S4A**	Varied TNF pulses in wild type vs. *iκbα*$^{-/-}$ vs. *a20*$^{-/-}$ *TNF Pulse Stimulation (1ng/ml)*	For each system (wild type, *iκbα*$^{-/-}$, *a20*$^{-/-}$) the model was run 180 times with TNF pulse lengths between 1 and 180 minutes. NF-κB activity is plotted on 3D color plots as described in Figure 2B. (Similar color plots are shown in Supplemental Figure 4A for IKK activity.)
4B **S4B**	Varied TNF pulses in wild type vs. *a20*$^{-/-}$. *5, 15, 45 minute TNF pulses (1ng/ml)*	The model was run with three TNF pulses for each system (wild type and *a20*$^{-/-}$). IKK (Supplemental Figure 4B) and NF-κB (Figure 4B) activities are plotted on 2 line plots.

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