Supporting Information

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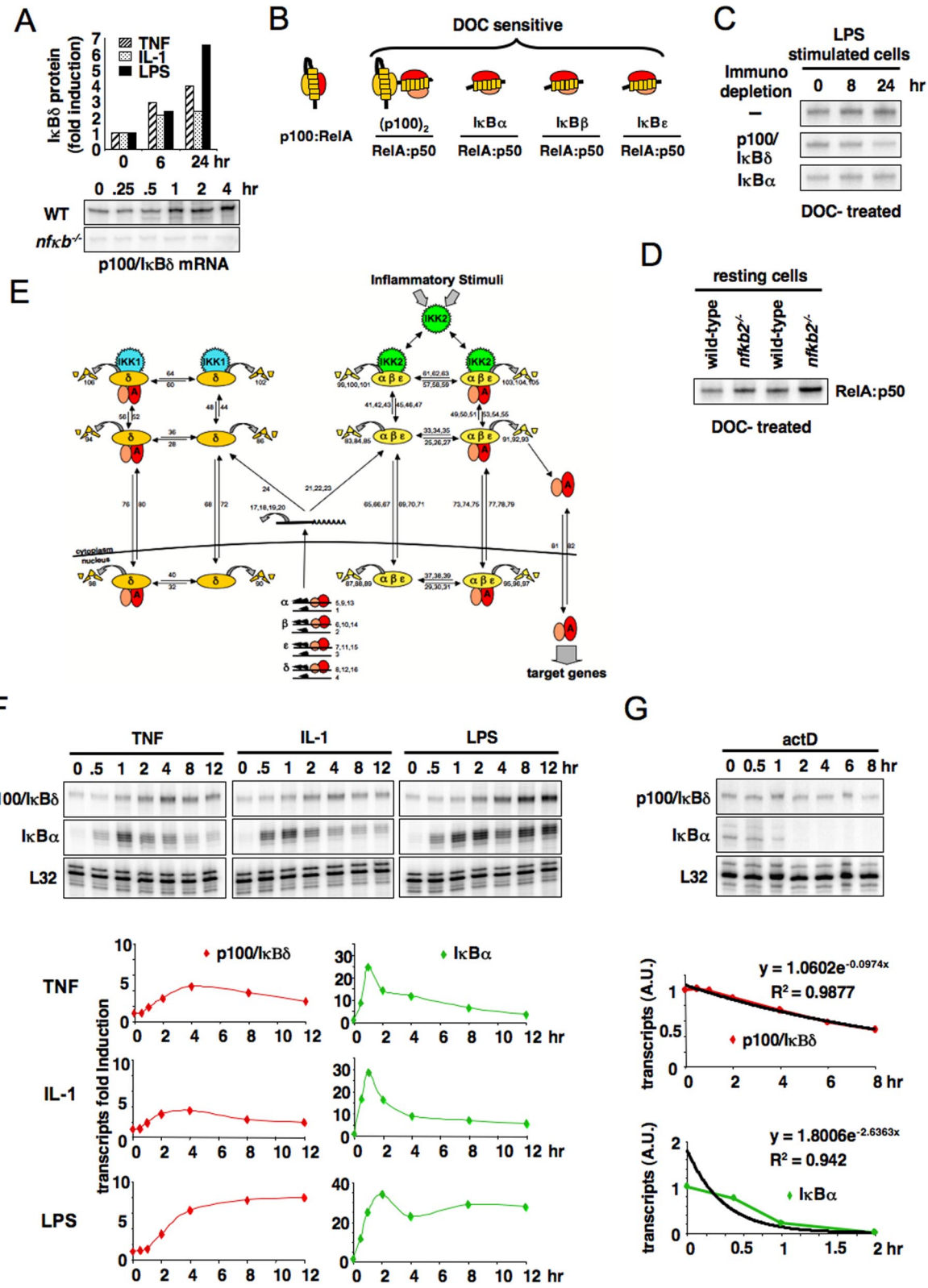

Fig. S1. (*A*) (*Top*) Quantitation of IκBδ protein expression in response to inflammatory signals. Band intensities were quantitated by phosphorimager, normalized to α-tubulin loading control and graphed relative to the signals in resting cells. (*Bottom*) RNAse Protection Assay revealing IκBδ mRNA induction upon chronic TFN (10 ng/ml) stimulation. RNA was harvested from wild type and nkκb deficient MEFs. (*B*) Diagram of different inhibited RelA-containing complexes, depicting RelA in red, p50 in orange, and ankyrin repeat domain (ARD)-containing proteins in yellow. IκBα, -β, -ε are shown to interact with NF-κB via the ARD. NF-κB/p100 protein is shown to engage in 2 distinct interactions with RelA; 1 mediated by the dimerization domain containing Rel homology domain (RHD) where ARD falls back in *cis* to mediate inhibition, the other by a free ARD resulting from $(p100)_2$ asymmetiric homo-dimerization. ARD-NF-κB-dimer *trans* interactions are sensitive to the detergent deoxycholate (DOC) whereas *cis* interactions are not. (*C*) Electrophoretic Mobility Shift Assay (EMSA) with MEF cytosolic extracts that were first immunodepleted with indicated IκB antibodies and then treated with deoxycholate to reveal NF-κB complexes associated with remaining IκB proteins. Immunodepletion of IκBδ antibody showed reduced deoxycholate-inducible NF-κB activity at late points of the chronic LPS (100 ng/mL) time course, whereas immunodepletion of IκBα resulted in smaller reductions in deoxycholate-inducible RelA:p50 at late time points (*Middle* and *Bottom*). Furthermore, no decrease of RelA activity was observed during LPS time course (*Top*), which indicates DOC-insensitive complexes, RelA:p100, are not significantly increased. Overall, it suggests that RelA is sequestered away by IκBδ, instead of p100, after LPS stimulation. (*D*) EMSA to reveal latent cytosolic NF-κB complexes. Cytosolic extracts harvested from resting wild-type and *nfkb2*$^{-/-}$ MEFs were treated with DOC and NF-κB DNA binding activities were analyzed by EMSA. *nfkb2*$^{-/-}$ cell extracts show elevated deoxycholate-inducible RelA:p50, indicating that DOC-insensitive complexes, RelA:p100, exist in wild-type cells. (*E*) Schematic of the reaction network. There are 100 reactions in the model, governed by 106 parameters, which describe the synthesis and degradation of IκBs, association and dissociation of IκBs, NF-κB, and IKK1 or IKK2, and cellular localization of IκBs and NF-κB. There are isoform-specific reaction rate constants for each of the 4 IκBs that are not explicitly shown. IκBα, IκBβ, and IκBε only associate with IKK2 whereas IκBδ only associates with IKK1. The numbers adjacent to each reaction arrow are the corresponding parameter numbers shown in Table S2. (*F*) RNase Protection Assay revealing IκBδ and IκBα mRNA induction kinetics upon inflammatory signals stimulation. RNA was harvested from wild-type MEFs stimulated with chronic TNF (1 ng/mL), IL-1 (1 ng/mL), or transient LPS (100 ng/mL) for 45 min. Signals were quantitated, normalized to ribosomal protein gene L32 (loading control) and graphed relative to the signals in resting cells. (*G*) Half-life measurements of IκBα and p100/IκBδ mRNAs by RNase Protection Assay. Wild-type MEFs were stimulated with LPS for 4 h and washed followed by actinomycinD treatment. RNA was then harvested at indicated time points. Signals were quantitated, normalized to L32 loading control and graphed relative to the signals in resting cells. Trend lines were generated by second order exponential curves that best fit the indicated data points.

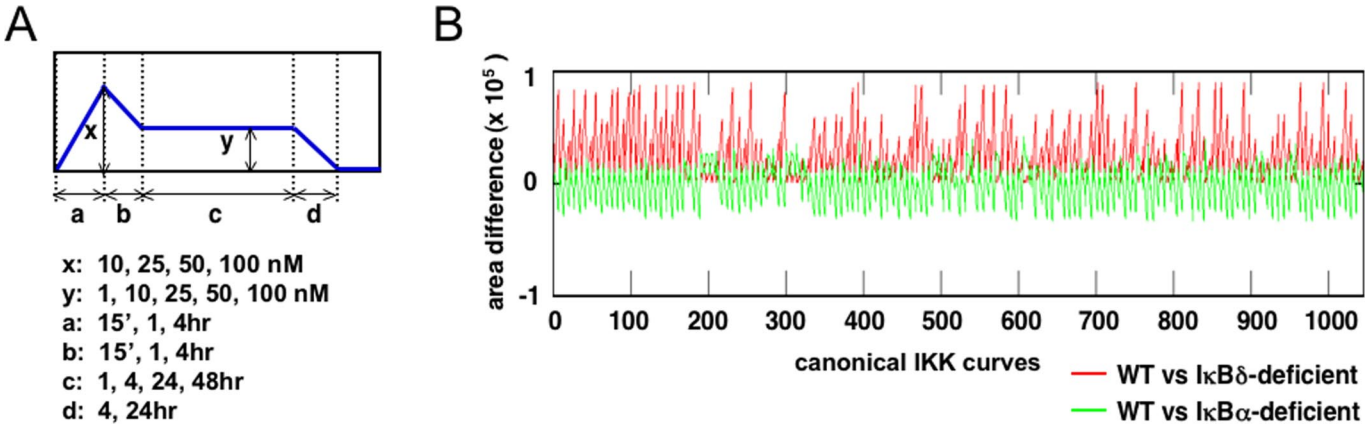

Fig. S2. (*A*) Schematic of the algorithm used to generate a library of canonical IKK curves. Each canonical IKK curve resulted from a specific combination of values for parameters *x*, *y*, and *a*–*d* as indicated: The rise of canonical IKK activity was determined by the duration a and peak level x; subsequent attenuation proceeded over time *b* to a second phase level *y*, which lasts for a duration *c*; this is followed by a return to base line over time d. (*B*) Computational phenotyping of the negative feedback regulators. The graph shows the difference of NF-κB activity between wild-type and mutant cells (difference in area under the curve) plotted against the corresponding canonical IKK activity curve.

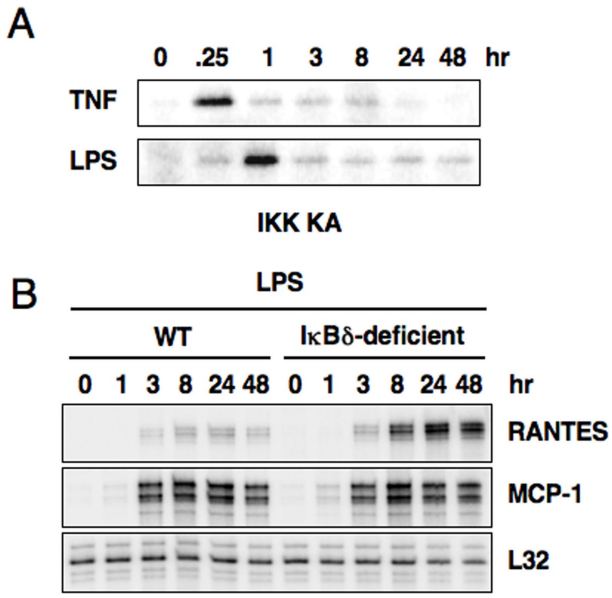

Fig. S3. (*A*) Replicates of IKK kinase activity assays (IKK KA) from MEFs stimulated with chronic TNF (1 ng/mL) or LPS (100 ng/mL). NEMO immunoprecipitates from cell extracts prepared at indicated times were reacted with GST-IκBα (1–54) protein in the presence of [^{32}]ATP. (*B*) Expression analysis of NF-κB target genes in response to LPS stimulation of MEFs. Wild-type and IκBδ-deficient cells were stimulated with chronic LPS (10 ng/mL). RNA was harvested at indicated time points and gene expression was analyzed by RPA.

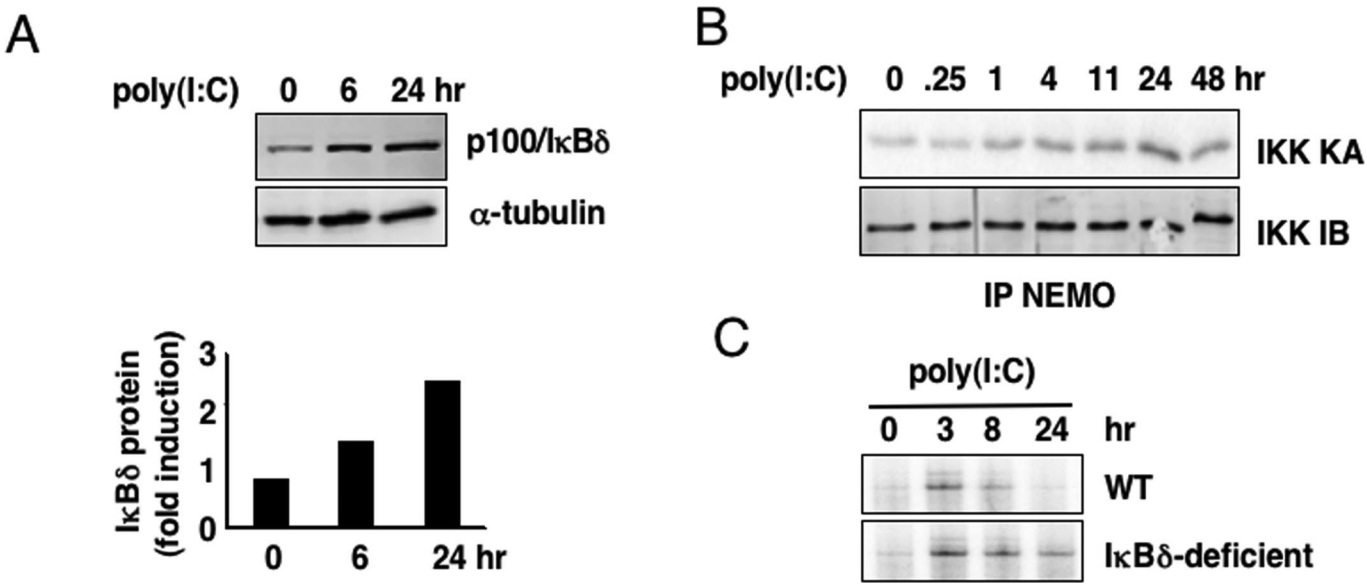

Fig. S4. (*A*) Western Blot analysis against IκBδ of whole cell extracts from 50 μg/mL poly(I:C)-stimulated wild-type MEFs. Equal loading was confirmed by immunoblotting against α-tubulin. Band intensities were quantitated, normalized to α-tubulin and graphed. (*B*) Canonical IKK activity profile was measured by IKK IP kinase assays from wild-type MEFs stimulated with poly(I:C) (50 μg/mL). NEMO immunoprecipitates from cell extracts prepared at indicated times were reacted with GST-IκBα (1–54) protein in the presence of [^{32}]ATP. Equal loading was confirmed by immunoblotting against IKK1. (*C*) NF-κB DNA binding activities induced by poly(I:C) (50 μg/mL) stimulation in wild-type and IκBδ-deficient MEFs were monitored by EMSA.

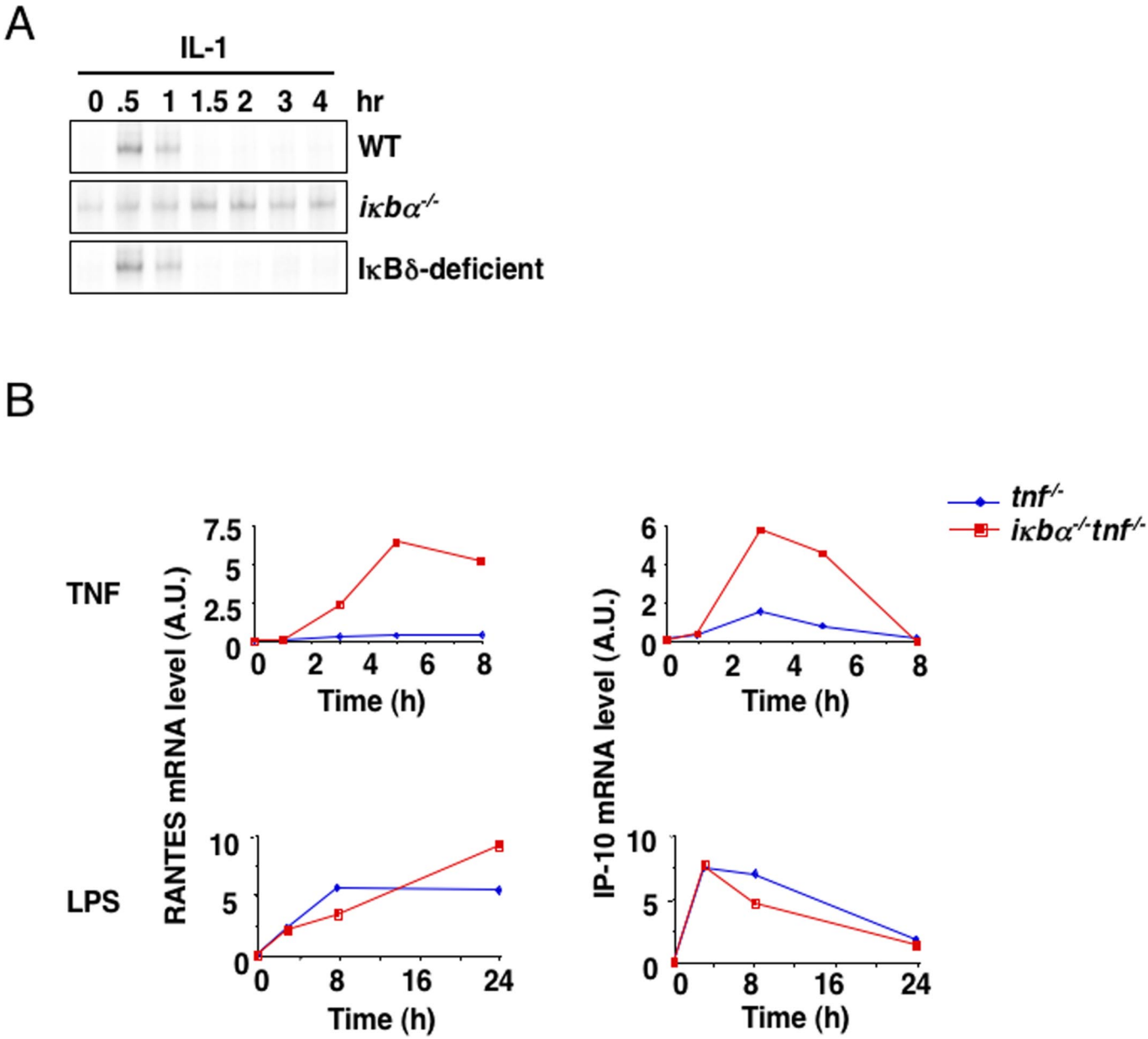

Fig. S5. (*A*) NF-κB DNA binding activities induced by 15 min transient IL-1 in wild-type and mutant MEFs were monitored by EMSA. (*B*) Quantitation of gene expression shown in Fig. 4*D*. Signals were quantitated, normalized to GAPDH loading control. Units derived were then multiplied by an appropriate number and graphed relative to the signals in resting cells.

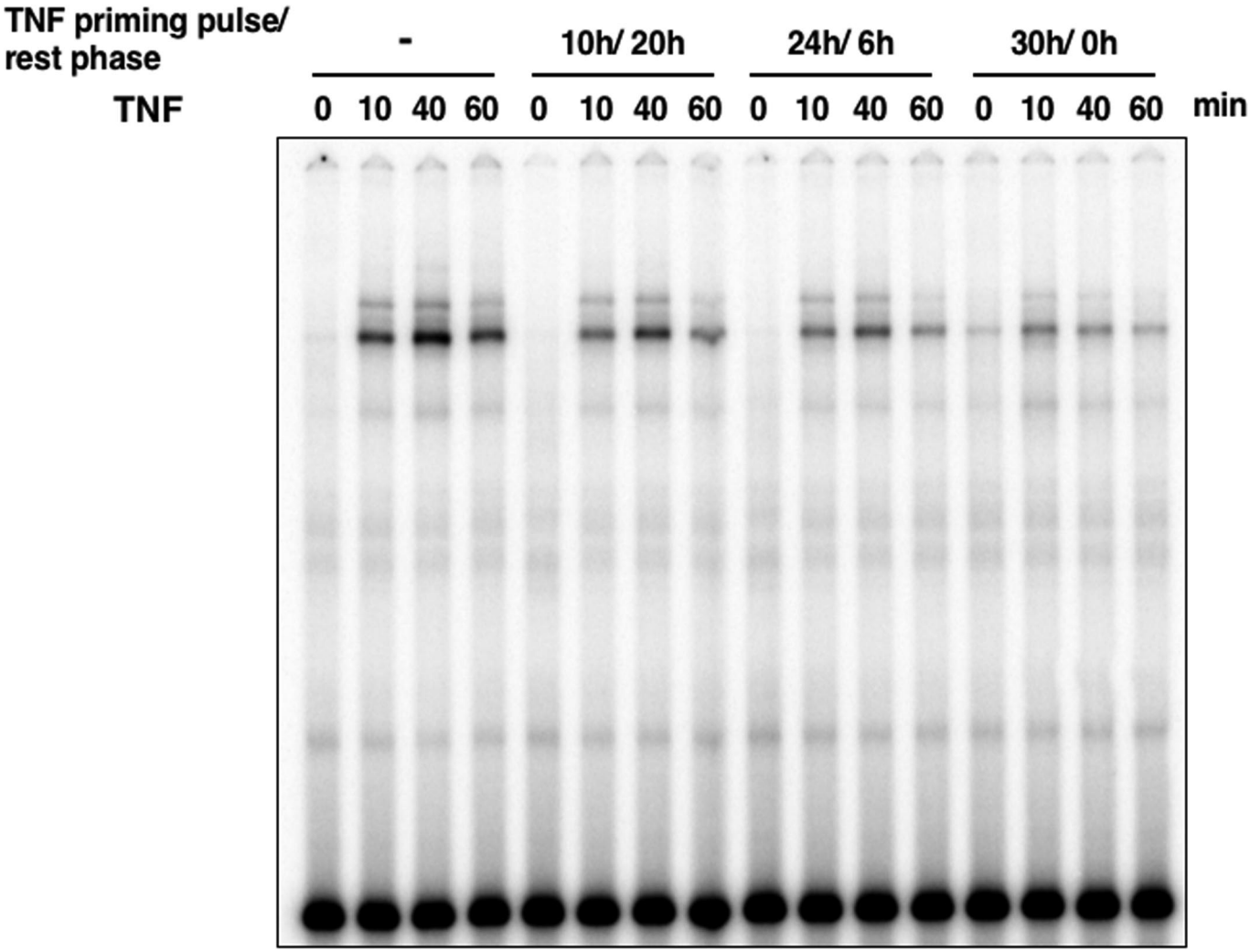

Fig. S6. Analysis of NF-κB activation in wild-type MEFs primed with TNF and rested for different periods of time as indicated. NF-κB activities in response to TNF were compared between naïve and primed cells by EMSA.

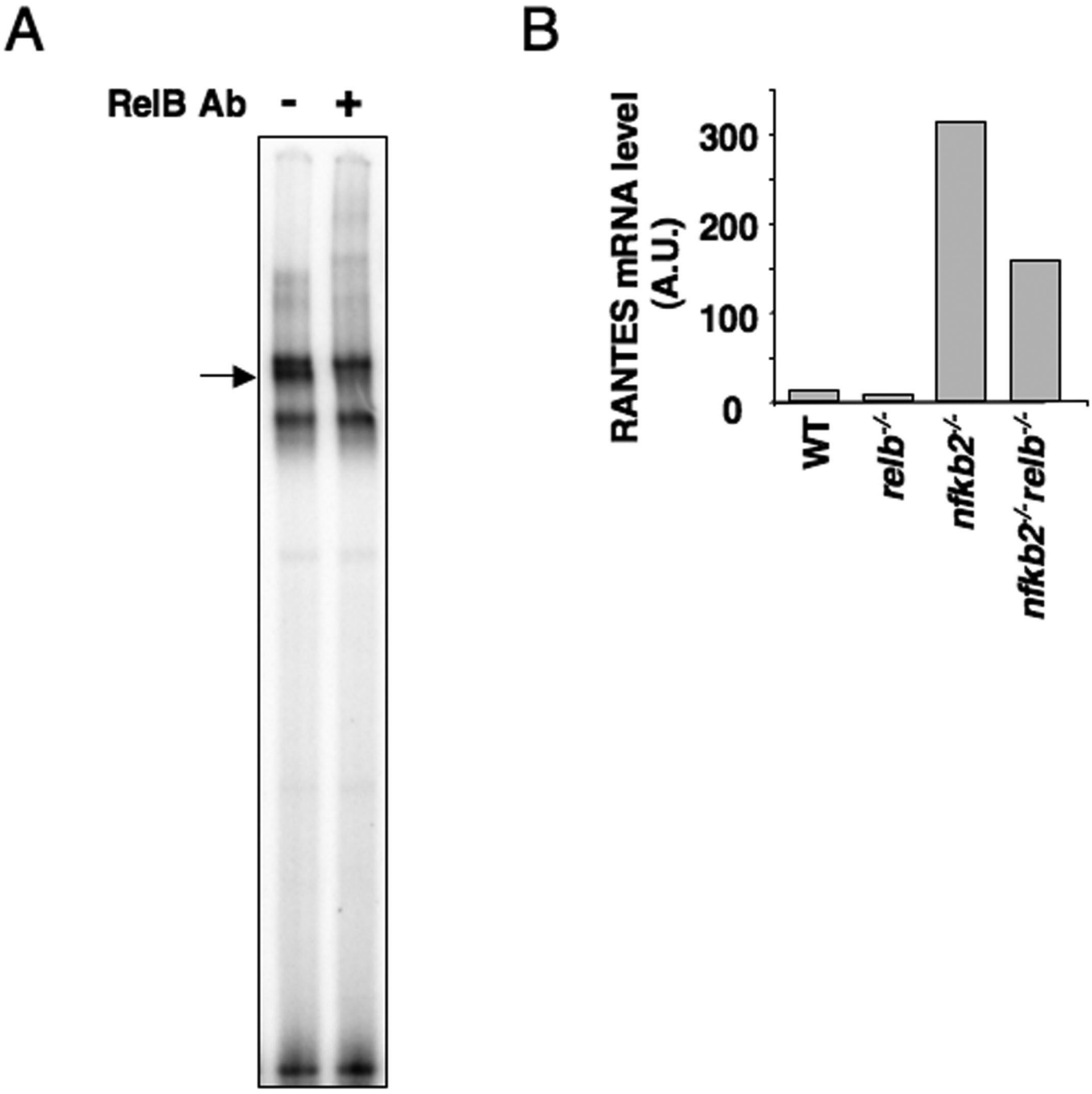

Fig. S7. (*A*) EMSA analysis to confirm specificity of RelB antibody. Nuclear extracts from *nfkb2*$^{-/-}$ MEFs stimulated with chronic LPS (100 ng/mL) were either immuno-depleted with RelB antibody or not before EMSA analysis. (*B*) Quantitation of RANTES gene expression in response to LPS. Wild-type and indicated gene-deficient cells were stimulated with chronic LPS (10 ng/mL, 48 h). RNA was harvested and gene expression was analyzed by RNase Protection Assay. Signals were quantitated, normalized to L32 loading control. Units derived were then multiplied by an appropriate number and graphed.

Table S1. Model species and initial concentrations

	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:IKK	IkBaIKK	0	Cytoplasm
6	IκBα:IKK:NF-κB	IkBaIKKNFkB	0	Cytoplasm
7	IκBα mRNA	IkBat	0	Cytoplasm
8	IκBβ	IkBb	0	Cytoplasm
9	IκBβ	IkBbn	0	Nucleus
10	IκBβ:NF-κB	IkBbNFkB	0	Cytoplasm
11	IκBβ:NF-κ B	IkBbNFkBn	0	Nucleus
12	IκBβ:IKK	IkBbIKK	0	Cytoplasm
13	IκBβ:IKK:NF-κB	IkBbIKKNFkB	0	Cytoplasm
14	IκBβ mRNA	IkBbt	0	Cytoplasm
15	IκBϵ	IkBe	0	Cytoplasm
16	IκBϵ	IkBen	0	Nucleus
17	IκBϵ:NF-kB	IkBeNFkB	0	Cytoplasm
18	IκBϵ:NF-kB	IkBeNFkBn	0	Nucleus
19	IκBϵ:IKK	IkBeIKK	0	Cytoplasm
20	IκBϵ:IKK:NF-κB	IkBeIKKNFkB	0	Cytoplasm
21	IκBϵ mRNA	IkBet	0	Cytoplasm
22	IKK2	IKK	0.1	Cytoplasm
23	NF-κB	NFkB	0	Cytoplasm
24	NF-κB	NFkBn	0.125	Nucleus
25	IKK1	IKK1	0.1	Cytoplasm
26	IκBδ	IkBd	0	Cytoplasm
27	IκBδ	IkBdn	0	Nucleus
28	IκBδ:NF-κB	IkBdNFkB	0	Cytoplasm
29	IκBδ:NF-κB	IkBdNFkBn	0	Nucleus
30	IκBδ:IKK1	IkBdIKK1	0	Cytoplasm
31	IκBδ:IKK1:NF-κB	IkBdIKK1NFkB	0	Cytoplasm
32	IκBδ mRNA	IkBdt	0	Cytoplasm

There are 32 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, IKK1, and IKK2 are conserved throughout the simulation (the sums of free, bound, active, and inactive forms in the cytoplasm and nucleus do not change). The initial concentrations of the remaining species are set to zero.

Table S2. Model parameters and biochemical rate constants

No.	Reaction	Parameter value	Category	Location	Source of parameter value
		IkB mRNA and protein synthesis reactions			
1	⇒ IkBat (constitutive)	2 E-4 min^{-1}	RNA synth.	—	mRNA synthesis parameters were derived from mRNA and Protein expression profiles measured by RNase Protection Assays (RPA) and Western Blots in wild type and NF-kB-deficient cells.
2	⇒ IkBbt (constitutive)	1 E-5 min^{-1}	RNA synth.	—	Refer to no. 1
3	⇒ IkBet (constitutive)	3 E-6 min^{-1}	RNA synth.	—	Refer to no. 1
4	⇒ IkBdt (constitutive)	1 E-7 min^{-1}	RNA synth.	—	Refer to no. 1
5	⇒ IkBat (induced by NFkBn)	6 μM^{-2} min^{-1}	RNA synth.	—	1
9		Hill Coefficient: 3.0			1
13		Delay: 0 min			2
6	⇒ IkBbt (induced by NFkBn)	0.25 μM^{-2} min^{-1}	RNA synth.	—	2
10		Hill Coefficient: 3.0			1
14		Delay: 37 min			2 and unpublished results
7	⇒ IkBet (induced by NFkBn)	0.5 μM^{-2} min^{-1}	RNA synth.	—	2
11		Hill Coefficient: 3.0			1
15		Delay: 37 min			2 and unpublished results
8	⇒ IkBdt (induced by NFkBn)	0.025 μM^{-2} min^{-1}	RNA synth.	—	mRNA synthesis parameters were derived from mRNA expression measurements in response to several inflammatory stimuli (SI Fig. 1F)
12		Hill Coefficient: 3.0			
16		Delay: 90 min			
17	IkBat ⇒	0.035 min^{-1}	RNA deg.	Cytoplasm	mRNA half-lives were determined by treating cells with Actinomycin-D and tracking the decay of the mRNA by RPA (SI Fig. 1G)
18	IkBbt ⇒	3 E-3 min^{-1}	RNA deg.	Cytoplasm	Refer to no. 17
19	IkBet ⇒	4 E-3 min^{-1}	RNA deg.	Cytoplasm	Refer to no. 17
20	IkBdt ⇒	2 E-3 min^{-1}	RNA deg.	Cytoplasm	Refer to no. 17
21	⇒ IkBa	0.25 min^{-1}	Prot. synth.	Cytoplasm	1
22	⇒ IkBb	0.25 min^{-1}	Prot. synth.	Cytoplasm	1
23	⇒ IkBe	0.25 min^{-1}	Prot. synth.	Cytoplasm	1
24	⇒ IkBd	0.25 min^{-1}	Prot. synth.	Cytoplasm	Assumed to be the same as for canonical IkBs
		IkB:IKK:NFkB association and dissociation reactions			
25	IkBa + NFkB ⇒ IkBaNFkB	30 μM^{-1} min^{-1}	Association	Cytoplasm	1
26	IkBb + NFkB ⇒ IkBbNFkB	30 μM^{-1} min^{-1}	Association	Cytoplasm	1
27	IkBe + NFkB ⇒ IkBeNFkB	30 μM^{-1} min^{-1}	Association	Cytoplasm	1
28	IkBd + NFkB ⇒ IkBdNFkB	30 μM^{-1} min^{-1}	Association	Cytoplasm	Assumed to be the same as for canonical IkBs
29	IkBan + NFkBn ⇒ IkBaNFkBn	30 μM^{-1} min^{-1}	Association	Nucleus	1
30	IkBbn + NFkBn ⇒ IkBbNFkBn	30 μM^{-1} min^{-1}	Association	Nucleus	1
31	IkBen + NFkBn ⇒ IkBeNFkBn	30 μM^{-1} min^{-1}	Association	Nucleus	1
32	IkBdn + NFkBn ⇒ IkBdNFkBn	30 μM^{-1} min^{-1}	Association	Nucleus	Assumed to be the same as for canonical IkBs
33	IkBaNFkB ⇒ IkBa + NFkB	6E-5 min^{-1}	Dissociation	Cytoplasm	1
34	IkBbNFkB ⇒ IkBb + NFkB	6E-5 min^{-1}	Dissociation	Cytoplasm	1
35	IkBeNFkB ⇒ IkBe + NFkB	6E-5 min^{-1}	Dissociation	Cytoplasm	1
36	IkBdNFkB ⇒ IkBd + NFkB	6E-5 min^{-1}	Dissociation	Cytoplasm	Assumed to be the same as for canonical IkBs
37	IkBaNFkBn ⇒ IkBan + NFkBn	6E-5 min^{-1}	Dissociation	Nucleus	1
38	IkBbNFkBn ⇒ IkBbn + NFkBn	6E-5 min^{-1}	Dissociation	Nucleus	1
39	IkBeNFkBn ⇒ IkBen + NFkBn	6E-5 min^{-1}	Dissociation	Nucleus	1
40	IkBdNFkBn ⇒ IkBdn + NFkBn	6E-5 min^{-1}	Dissociation	Nucleus	Assumed to be the same as for canonical IkBs
41	IkBa + IKK2 ⇒ IkBaIKK2	1.35 μM^{-1} min^{-1}	Association	Cytoplasm	1
42	IkBb + IKK2 ⇒ IkBbIKK2	0.36 μM^{-1} min^{-1}	Association	Cytoplasm	1
43	IkBe + IKK2 ⇒ IkBeIKK2	0.54 μM^{-1} min^{-1}	Association	Cytoplasm	1
44	IkBd + IKK1 ⇒ IkBdIKK1	0.54 μM^{-1} min^{-1}	Association	Cytoplasm	Assumed to be the same as for IkBe but pertaining to IKK1
45	IkBaIKK2 ⇒ IkBa + IKK2	0.075 min^{-1}	Dissociation	Cytoplasm	1
46	IkBbIKK2 ⇒ IkBb + IKK2	0.105 min^{-1}	Dissociation	Cytoplasm	1
47	IkBeIKK2 ⇒ IkBe + IKK2	0.105 min^{-1}	Dissociation	Cytoplasm	1
48	IkBdIKK1 ⇒ IkBd + IKK1	0.105 min^{-1}	Dissociation	Cytoplasm	Assumed to be the same as for IkBe but pertaining to IKK1
49	IkBaNFkB + IKK2 ⇒ IkBaIKK2NFkB	11.1 μM^{-1} min^{-1}	Association	Cytoplasm	1
50	IkBbNFkB + IKK2 ⇒ IkBbIKK2NFkB	2.88 μM^{-1} min^{-1}	Association	Cytoplasm	1
51	IkBeNFkB + IKK2 ⇒ IkBeIKK2NFkB	4.2 μM^{-1} min^{-1}	Association	Cytoplasm	1
52	IkBdNFkB + IKK1⇒ IkBdIKK1NFkB	4.2 μM^{-1} min^{-1}	Association	Cytoplasm	Assumed to be the same as for IkBe but pertaining to IKK1
53	IkBaIKK2NFkB ⇒ IkBaNFkB + IKK2	0.075 min^{-1}	Dissociation	Cytoplasm	1
54	IkBbIKK2NFkB ⇒ IkBbNFkB + IKK2	0.105 min^{-1}	Dissociation	Cytoplasm	1

No.	Reaction	Parameter value	Category	Location	Source of parameter value
55	IkBeIKK2NFkB ⇒ IkBeNFkB + IKK2	0.105 min^{-1}	Dissociation	Cytoplasm	1
56	IkBdIKK1NFkB⇒ IkBdNFkB + IKK1	0.105 min^{-1}	Dissociation	Cytoplasm	Assumed to be the same as for IkBe but pertaining to IKK1
57	IkBaIKK2 + NFkB ⇒ IkBaIKK2NFkB	30 μM^{-1} min^{-1}	Association	Cytoplasm	1
58	IkBbIKK2 + NFkB ⇒ IkBbIKK2NFkB	30 μM^{-1} min^{-1}	Association	Cytoplasm	1
59	IkBeIKK2 + NFkB ⇒ IkBeIKK2NFkB	30 μM^{-1} min^{-1}	Association	Cytoplasm	1
60	IkBdIKK1 + NFkB⇒ IkBdIKK1NFkB	30 μM^{-1} min^{-1}	Association	Cytoplasm	Assumed to be the same as for canonical IkBs
61	IkBaIKK2NFkB ⇒ IkBaIKK2 + NFkB	6E-5 min^{-1}	Dissociation	Cytoplasm	1
62	IkBbIKK2NFkB ⇒ IkBbIKK2 + NFkB	6E-5 min^{-1}	Dissociation	Cytoplasm	1
63	IkBeIKK2NFkB ⇒ IkBeIKK2 + NFkB	6E-5 min^{-1}	Dissociation	Cytoplasm	1
64	IkBdIKK1NFkB⇒ IkBdIKK1 + NFkB	6E-5 min^{-1}	Dissociation	Cytoplasm	Assumed to be the same as for canonical IkBs
		IkB and NFkB cellular localization reactions			
65	IkBa ⇒ IkBan	0.09 min^{-1}	Import	—	1
66	IkBb ⇒ IkBbn	0.009 min^{-1}	Import	—	1
67	IkBe ⇒ IkBen	0.045 min^{-1}	Import	—	1
68	IkBd ⇒ IkBdn	0.045 min^{-1}	Import	—	Assumed to be the same as for IkBe
69	IkBan ⇒ IkBa	0.012 min^{-1}	Export	—	1
70	IkBbn ⇒ IkBb	0.012 min^{-1}	Export	—	1
71	IkBen ⇒ IkBe	0.012 min^{-1}	Export	—	1
72	IkBdn ⇒ IkBd	0.012 min^{-1}	Export	—	Assumed to be the same as for canonical IkBs
73	IkBaNFkB ⇒ IkBaNFkBn	0.276 min^{-1}	Import	—	1
74	IkBbNFkB ⇒ IkBbNFkBn	0.0276 min^{-1}	Import	—	1
75	IkBeNFkB ⇒ IkBeNFkBn	0.138 min^{-1}	Import	—	1
76	IkBdNFkB ⇒ IkBdNFkBn	0.276 min^{-1}	Import	—	Assumed to be the same as for IkBa
77	IkBaNFkBn ⇒ IkBaNFkB	0.828 min^{-1}	Export	—	1
78	IkBbNFkBn ⇒ IkBbNFkB	0.414 min^{-1}	Export	—	1
79	IkBeNFkBn ⇒ IkBeNFkB	0.414 min^{-1}	Export	—	1
80	IkBdNFkBn ⇒ IkBdNFkB	0.414 min^{-1}	Export	—	Assumed to be the same as for canonical IkBs
81	NFkB ⇒ NFkBn	5.4 min^{-1}	Import	—	1
82	NFkBn ⇒ NFkB	0.0048 min^{-1}	Export	—	1
		IkB protein degradation reactions			
83	IkBa ⇒	0.12 min^{-1}	Prot. deg.	Cytoplasm	3
84	IkBb ⇒	0.18 min^{-1}	Prot. deg.	Cytoplasm	3
85	IkBe ⇒	0.18 min^{-1}	Prot. deg.	Cytoplasm	3
86	IkBd ⇒	1.4E-3 min^{-1}	Prot. deg.	Cytoplasm	The IkBd half-life was measured by quantitative Western blot (unpublished results).
87	IkBan ⇒	0.12 min^{-1}	Prot. deg.	Nucleus	3
88	IkBbn ⇒	0.18 min^{-1}	Prot. deg.	Nucleus	3
89	IkBen ⇒	0.18 min^{-1}	Prot. deg.	Nucleus	3
90	IkBdn ⇒	1.4E-3 min^{-1}	Prot. deg.	Nucleus	*Refer to #86*
91	IkBaNFkB ⇒ NFkB	6E-5 min^{-1}	Prot. deg.	Cytoplasm	3
92	IkBbNFkB ⇒ NFkB	6E-5 min^{-1}	Prot. deg.	Cytoplasm	3
93	IkBeNFkB ⇒ NFkB	6E-5 min^{-1}	Prot. deg.	Cytoplasm	3
94	IkBdNFkB ⇒ NFkB	6E-5 min^{-1}	Prot. deg.	Cytoplasm	Assumed to be the same as for canonical IkBs
95	IkBaNFkBn ⇒ NFkBn	6E-5 min^{-1}	Prot. deg.	Nucleus	3
96	IkBbNFkBn ⇒ NFkBn	6E-5 min^{-1}	Prot. deg.	Nucleus	3
97	IkBeNFkBn ⇒ NFkBn	6E-5 min^{-1}	Prot. deg.	Nucleus	3
98	IkBdNFkBn ⇒ NFkBn	6E-5 min^{-1}	Prot. deg.	Nucleus	Assumed to be the same as for canonical IkBs
99	IkBaIKK2 ⇒ IKK2	1.8E-3 min^{-1}	Prot. deg.	Cytoplasm	1
100	IkBbIKK2 ⇒ IKK2	6E-4 min^{-1}	Prot. deg.	Cytoplasm	1
101	IkBeIKK2 ⇒ IKK2	1.2E-3 min^{-1}	Prot. deg.	Cytoplasm	1
102	IkBdIKK1 ⇒ IKK1	1.2E-3 min^{-1}	Prot. deg.	Cytoplasm	Assumed to be the same as for IkBe
103	IkBaIKK2NFkB ⇒ IKK2 + NFkB	0.36 min^{-1}	Prot. deg.	Cytoplasm	1
104	IkBbIKK2NFkB ⇒ IKK2 + NFkB	0.12 min^{-1}	Prot. deg.	Cytoplasm	1
105	IkBeIKK2NFkB ⇒ IKK2 + NFkB	0.18 min^{-1}	Prot. deg.	Cytoplasm	1
106	IkBdIKK1NFkB ⇒ IKK1 + NFkB	0.18 min^{-1}	Prot. deg.	Cytoplasm	Assumed to be the same as for IkBe

Parameter identifiers (column 1 and SI Fig.1E) are related to reaction descriptions and reaction rate constants.

1. Werner SL, Barken D, Hoffmann A (2005) Stimulus specificity of gene expression programs determined by temporal control of IKK activity. *Science* 309:1857–1861.
2. Kearns JD, Basak S, Werner SL, Huang CS, Hoffmann A (2006) IkappaBepsilon provides negative feedback to control NF-kappaB oscillations, signaling dynamics, and inflammatory gene expression. *J Cell Biol* 173:659–664.
2. O'Dea EL, et al. (2007) A homeostatic model of IkappaB metabolism to control constitutive NF-kappaB activity. *Mol Syst Biol* 3:111.