IκBβ enhances the generation of the low-affinity NFκB/RelA homodimer

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The NFκB family of dimeric transcription factors regulate inflammatory and immune responses. While the dynamic control of NFκB dimer activity via the IκB–NFκB signalling module is well understood, there is little information on how specific dimer repertoires are generated from Rel family polypeptides. Here we report the iterative construction—guided by in vitro and in vivo experimentation—of a mathematical model of the Rel-NFκB generation module. Our study reveals that IκBβ has essential functions within the Rel-NFκB generation module, specifically for the RelA:RelA homodimer, which controls a subset of NFκB target genes. Our findings revise the current dogma of the three classical, functionally related IκB proteins by distinguishing between a positive ‘licensing’ factor (IκBβ) that contributes to determining the available NFκB dimer repertoire in a cell’s steady state, and negative feedback regulators (IκBα and –e) that determine the duration and dynamics of the cellular response to an inflammatory stimulus.
The NFκB family of dimeric transcription factors are known to control the development, maturation and responses of the immune system comprising diverse cell types. Resting, unstimulated cells contain a latent pool of NFκB dimers in the cytosol that are stoichiometrically associated with their inhibitors, the IκBs. NFκB transcription factors are produced by homo- or heterodimerization from a pool of five Rel homology domain-containing proteins (RelA, RelB, cRel, p50 and p52). The IκB family comprises several isoforms including the classical IκBα, IκBβ, IκBε proteins and the IκBγ and IκBδ activities contained within the higher molecular weight IκBκsome. Combinatorial dimerization and IκB–NFκB interactions are hallmarks of the IκB–NFκB signalling system.

The coordinated functions of IκB proteins in controlling the dynamics of NFκB activity have been studied systematically using a combined experimental and mathematical modelling approach. Specifically, mathematical modelling recapitulates the key mechanisms, such as stimulus-responsive, IκB kinase (IκK)-dependent degradation of IκBs, release of nuclear NFκB DNA-binding activity, as well as subsequent attenuation via the negative feedback regulators IκBα, IκBε, A20 and IκBκ (ref. 4). Interestingly, these studies did not identify a critical function for IκBβ, and IκBβ-deficient mice and cells show attenuated, not increased inflammatory responses.

In contrast, little recent progress has been reported on how NFκB dimers are generated. Early studies led to an appreciation that the NFκB transcription factor family consists of up to 15 possible dimers, and that different dimers are detectable in different cell types. In fact, the NFκB dimer repertoire changes dramatically during cell differentiation; for example, B-cell lines with early lineage markers contain primarily RelA:p50, while those with later lineage markers contain primarily cRel:p50 dimers. In murine embryonic fibroblasts (MEFs), the RelA:p50 heterodimer and the RelA:RelA homodimer have been observed and shown to be responsible for the expression of distinct target genes. Indeed, despite its much lower abundance in fibroblasts, the RelA:RelA homodimer was shown to have nonredundant functions for a subset of NFκB target genes, with the specificity being mediated by κB-site DNA sequences as well as transcriptional co-activators. However, surprisingly little is known about the mechanisms that control the formation of these distinct NFκB dimers that are critical for mediating gene-specific expression control. Indeed, we presently lack a quantitative understanding of the most fundamental processes of monomer synthesis and subsequent dimer formation.

Here we have employed mathematical modelling and experimental approaches iteratively to study the mechanisms that control the fibroblast-specific NFκB dimer repertoire. Guided by a model based on first principles of protein–protein interactions, and using in vitro biophysical interaction measurement, we found that we can only account for the in vivo observations of low-affinity dimers when we considered additional mechanisms. Specifically, combined experimental and computational studies revealed that IκBβ functions as a positive regulator within the Rel-NFκB dimer generation module, and is essential for the formation of RelA:RelA homodimers. In contrast, IκBα is the key regulator of the dynamics of NFκB activity, not only of RelA:p50 but also of RelA:RelA. We conclude that the classical IκBs actually fall into two classes; whereas IκBβ and -ε primarily function within the IκB–NFκB signalling module that is responsive to inflammatory stimuli, IκBβ primarily functions within the Rel-NFκB dimer generation module. The resulting model not only recapitulates experimental observations, but also explains the contrasting phenotypes of mice deficient for each of the classical IκBs.

**Results**

**NFκB dimerization affinities and monomer competition.** To address the molecular basis for the generation of a MEF-specific repertoire of NFκB dimers within the so-called ‘Rel-NFκB dimer generation module’, we first considered the fundamental principles pertaining to the generation of homo- and heterodimers from the classical NFκB proteins p50 (50) and RelA (A), which are present in all differentiated human cells, and which show distinct gene-expression specificities. Dimer abundance may be thought of as a function of monomer synthesis ($m_1,m_2$) and degradation ($m_1,m_2$), and combinatorial dimer association ($d_1,d_2,d_3$) and dissociation ($d_1,d_2,d_3$) rate constants (Fig. 1a, Supplementary Table 1, Supplementary Note 1). As RNAseq data show very similar absolute abundances of the RelA and p50-encoding mRNAs in wild-type MEFs (Supplementary Fig. 1a), the synthesis rates of the protein monomers are assumed to be equal in the model. As Rel proteins are obligate dimers, monomers are incompletely folded and more rapidly degraded in vivo than when dimerized ($d_1,d_2,d_3$).

Dimerization affinities are the key determinants of cellular homo- and heterodimer abundances. We simulated the abundance of RelA:RelA (A:A) and RelA:p50 (A:50) dimers as a function of those dimer affinities keeping the p50:p50 dimer affinity constant at 30 nM (Fig. 1b), 3 and 300 nM (Supplementary Fig. 1b). Whereas p50:p50 dimerization affinity did not affect A:A or A:50 abundances over that range, the simulations do illustrate an interdependence between A:A and A:50 formation, whereby p50 competes for RelA monomers and may thus inhibit A:A generation. To test whether monomer competition occurs in cells, we compared nfkbi $^{-1}$/− (which lack p50) and wild-type MEFs and found an increase in the abundance of the A:A homodimers. These data support the concept that monomer competition is physiologically relevant and tends to diminish the abundances of weaker affinity dimers (Fig. 1c–e).

To parameterize the model, we next measured dimer affinities with biophysical techniques. Homodimer affinities were measured by analytical ultracentrifugation using purified recombinant RelA and p50 dimerization domains (Supplementary Fig. 1c). The heterodimer affinity was deduced from quantitative gel filtration experiments that provided the ratios of dimer abundances (Supplementary Fig. 1d–f). We found remarkably disparate affinities: whereas the interactions by p50 proteins were found within the expected range (5–40 nM), the A:A homodimer affinity was in the μM range (Fig. 2a). Employing these rate constants in the kinetic model resulted in the prediction that the A:A homodimers would have exceedingly low abundance (<4% of total RelA-containing dimers) because of a weak A:A affinity and the above-described monomer competition. However, testing this prediction experimentally, we found that A:A homodimers were readily detectable in cells amounting to about a quarter of tumour-necrosis factor (TNF)-inducible NFκB activities (Fig. 2b). Thus, the mismatch between model prediction and experimental results suggested that there might be additional mechanisms controlling dimer abundance that are not included in this version of the kinetic model.

**Interaction affinities between IκB isoforms and NFκB dimers.** Given that IκBs associate along a large dimerization interface, and NFκBs interacting with IκBs protect the latter from degradation, we considered the possibility that IκBs may stabilize RelA-containing NFκB dimers. (Classical IκBs do not interact with p50:p50 (ref. 15).) To test this idea, we extended the mathematical model by including IκB synthesis ($i_1$) and degradation ($i_2$) and its interaction with A:A homodimers and...
A:50 heterodimers \((d_{i1}, d_{i-1}, d_{i2}, d_{i-2})\); Fig. 3a, Supplementary Table 2, Supplementary Note 2). In the resulting model, NFκB dimer abundances are then also a function of IκB dimer interactions and the interaction affinities between NFκB dimers and IκB proteins. These interactions are thought to be tight;\(^{12,16,17}\) however, systematic measurements using biophysical methods are hampered by the challenge of expressing and purifying full-length recombinant proteins that are not fully folded in the apof orm and have a tendency to aggregate.

To address this challenge, we developed an approach to derive \textit{in vivo} IκB–NFκB affinities. The fact that IκB\(_{\alpha}\), IκB\(_{\beta}\) and IκB\(_{d}\) protein abundances in MEFs deficient in classical NFκB dimers (\(rela^{-/-}\ crel^{-/-}\ nfκb\(_{1}\)^{-/-}\)) were much more reduced than their corresponding mRNA levels (Fig. 3b) indicated the degree to which the interaction with NFκB stabilized the half-life of the three classical IκBs as established by previous reports\(^{12,14}\). We reasoned that measurements of relative abundances of IκB proteins in mutant cells with alternate NFκB dimer repertoires could be used to infer \textit{in vivo} IκB–NFκB dimer affinities.

To distinguish IκB interaction affinities for the A:50 and A:A dimers, we produced composite NFκB knockout MEFs (\(relb^{-/-}\ nfκb\(_{2}\)^{-/-}\ nfκb\(_{1}\)^{-/-}\ crel^{-/-}\)) in which RelA is the sole NFκB protein family member, and RelA-deficient MEFs, which do not contain A:A homodimers but contain the partially compensating \(cRel\)-NFκB heterodimer in place of A:50 (refs 7,18; Supplementary Fig. 2a,b). Using these cells, the experimentally determined IκB\(_{\alpha}/IκB\(_{\beta}\) abundance ratios (Fig. 3c) constrained the acceptable ranges of IκB–NFκB affinities via model parameter fitting procedures (Fig. 4a). For example, in A:50-deficient cells, the IκB\(_{\alpha}\) abundance was reduced to 35 ± 8% of that in wild-type cells, constraining the IκB\(_{\alpha}/IκB\(_{\beta}\)-A:A and IκB\(_{\alpha}/IκB\(_{\beta}\)-A:50 affinity to a narrow band within the two-dimensional parameter space (top left panel). In contrast, IκB\(_{\beta}\) abundance was hardly affected (76 ± 24% of the wild-type cells) and provided less constraint on the acceptable affinities of IκB\(_{\beta}/IκB\(_{\alpha}\)-A:A and IκB\(_{\beta}/IκB\(_{\alpha}\)-A:50 interactions on the basis of the particular parameter landscape (top right panel). Similarly, IκB\(_{\alpha}\) and IκB\(_{\beta}\) abundance measurements in A:A-deficient cells provided additional constraints (bottom panels). The overlap of these acceptable ranges for each IκB in both NFκB knockout conditions determined not a single parameter set, but rather a narrower range of parameters that can account for all available experimental data.

As the NFκB dimer affinity and IκB abundance measurements are associated with experimental error, we sought to characterize the resulting uncertainty about the true IκB–NFκB affinities. To do so, we calculated a probability for each IκB abundance on the basis of a twofold variance around the mean of NFκB affinity measurements. Then, we compared these values to the experimentally determined distributions (defined by mean and s.d.) of IκB abundances. This allowed us to determine the likelihood that a given pair of IκB–A:A and IκB–A:50 affinities is true based on all available experimental data along with its associated uncertainty (Fig. 4b). This analysis allowed us to define the ranges of probable IκB-dimer affinities and it demonstrated that IκB\(_{\beta}\) was consistently predicted to have a clear preference for A:A over A:50 (>100-fold). We chose seven parameter sets as

**Figure 1** | Monomer competition reduces the abundance of the RelA homodimer. (a) A schematic of a simple model depicting the dimerization of NFκB RelA and p50 polypeptides. \(m_1\) and \(m_2\) are synthesis rate constants, \(m_{-1}\) and \(m_{-2}\) are degradation rate constants. \(d_1\), \(d_2\) and \(d_3\) are dimer association rate constants, while \(d_{-1}\), \(d_{-2}\) and \(d_{-3}\) are dimer dissociation rate constants. \(K_d\) is the dimer degradation rate constants. (b) Heat maps of protein abundances of A:A and A:50 dimers computationally simulated as a function of the dimer affinities. (Kd). These were generated by the model schematized in a. (c) Electrophoretic mobility shift assays (EMSA) with nuclear extracts prepared from TNF-stimulated (30 min) wild-type MEFs. (d) EMSA with nuclear extracts prepared from TNF-stimulated (30 min) wild-type and A:50−/− MEFs. (e) Bar graph comparison of the abundances of A:A NFκB dimers wild-type and A:50−/− relative to the wild-type A:50 bands (100%) from d.

**Figure 2** | Biophysical kinetic model underestimates the abundance of the RelA homodimer. (a) Summary table of dimer affinities as measured with AUC and quantitative gel-filtration analysis of purified recombinant proteins (Supplementary Fig. 1b–e). The A:A homodimer affinity is lower than those of p50-containing dimers. (b) The bar graph is a quantitation from Fig. 1 showing a comparison of model predicted (blue) and experimentally measured (red) relative abundances of A:A and A:50 dimers.
representatives of the probable and improbable parameter spaces governing IκBα and IκBβ interactions (Fig. 4b, Supplementary Table 5) in order to ascertain their likelihood in further cell biological assays.

Testing the model of IκBβ controlling the RelA homodimer.

Using the combination of probable and improbable parameter sets (Supplementary Table 5) for IκB–NFκB dimer affinities within the experimentally determined ranges, we parameterized a mathematical model to account for the generation of distinct RelA dimers (Fig. 5a, Supplementary Table 3, Supplementary Note 3). We developed three predictions that depend on high IκBβ preference for the A:A homodimer and tested them experimentally. First, we used the model to calculate the basal abundance of RelA protein in cells containing only RelA (relb<sup>-/-</sup> nfkb2<sup>-/-</sup> nfkb1<sup>-/-</sup> crel<sup>-/-</sup>) under two regimes, probable and improbable affinity predictions (Supplementary Table 5). We found that most probable affinity sets predicted a lower decrease (~1.5- to 2-fold) in RelA protein levels than most improbable affinity sets (Fig. 5b). Indeed, immunoblot analysis showed only a small decrease (~1.5-fold) in RelA protein (Fig. 5c).

Second, we used the model to simulate the steady-state abundances of IκB-bound NFκB dimers in wild-type cells; this resulted in the prediction that the majority of A:A homodimers are associated with IκBβ for the probable, but not the improbable affinity sets (Fig. 5d). Further, the majority of A:50 dimers were predicted to be associated with IκBβ, but in some, the primary interaction partner was IκBβ (Fig. 5d). To test this prediction we prepared cytoplasmic extracts from unstimulated MEFs that contained latent RelA–NFκB complexes. The ionic detergent deoxycholate is known to disrupt IκB–NFκB interactions while leaving the dimer intact and capable of binding DNA<sup>19</sup>, thereby revealing both latent RelA homo- and heterodimers (Fig. 5e, lane 2). By prior immunodepleting IκBα or IκBβ, we found that IκBβ (>50%) is indeed the primary interaction partner for the A:A homodimer (lane 6), whereas IκBζ depletion left the A:A homodimer largely intact (lane 4). In addition, we found that IκBζ is the primary interaction partner (>50%) for the A:50 heterodimer (lane 4), although some A:50 is bound to IκBβ as well (lane 6). On the basis of these data, we were able to narrow the probable parameter range by excluding parameter sets that did not satisfy the experimentally determined IκB–NFκB interactions (Supplementary Table 5).

Finally, we asked whether IκBβ is in fact required for A:A homodimer generation. Computational simulations with the probable affinity sets predicted a dramatic decrease (~20% of wild type) in A:A homodimer abundance in IκBβ-deficient but
are in red, while the least probable affinities are in dark blue. Selected probable and improbable parameter values are indicated (white letters and numbers, indicated in the colour scale) were calculated using the distribution experimental measurements, defined by the mean and s.d. (Figs 2a, 3c and 4a); allowable IκB dimer-deficient cells relative to WT. (A representative data figure is shown in Fig. 3c.) Experimentally determined abundances constrained the range of cells deficient for the indicated NFκB target genes (Supplementary Fig. 3). Notably, two NFκB-deficient cell lines showed a marked reduction in IκB abundance as revealed by deoxycholate treatment of cytoplasmic extracts, whereas expression of RelA:p50 target genes was not reduced but elevated instead (Supplementary Fig. 3).

In p50-deficient cells, compensation by p52 is observed but their relationship to each other by simulating the effect of IκB deficiency in the context of p50-containing or p50-deficient cells. In p50-deficient cells, compensation by p52 is observed but remains incomplete; in our simulations we account for that with a 30-fold reduction in p50 synthesis rates (qualitatively similar results were obtained with a range of synthesis rates; Fig. 6a). We find that loss of IκB in p50-deficient cells result in lower A:A abundance, although the A:A homodimer remains detectable in contrast to results in p50-containing cells, in which A:A abundance was more IκB-dependent (Fig. 5f,g).

Figure 4 | Deriving probable IκB–NFκB interaction affinities from experimental data. (a) Surface plots of indicated IκB abundances (α or β) in cells deficient for the indicated NFκB dimer (RelA:p50 heterodimer-deficient, top; RelA:RelA homodimer-deficient, bottom) over the indicated ranges of IκB–NFκB affinities, as predicted by the model schematized in Fig. 3a. Middle, bar graphs of the experimentally IκB abundances in the indicated NFκB dimer-deficient cells relative to WT. (A representative data figure is shown in Fig. 3c.) Experimentally determined abundances constrained the range of allowable IκB–NFκB affinities in the surface plots, indicated by shadows on the xy plane. (b) Heat maps of probable IκB–NFκB affinities. Probabilities (indicated in the colour scale) were calculated using the distribution experimental measurements, defined by the mean and s.d. (Figs 2a, 3c and 4a); they describe the relative likelihood that an indicate parameter set if correct based on available experimental data. The most probable IκB–NFκB affinities are in red, while the least probable affinities are in dark blue. Selected probable and improbable parameter values are indicated (white letters and numbers, listed in Supplementary Table 5), and were used for model predictions in Fig. 5.

IκBβ counters monomer competition and rescues RelA homodimer.

Our work thus far has identified two system properties that control the RelA:RelA abundance: monomer competition and IκBβ-mediated stabilization. These mechanisms have opposing effects on RelA:RelA homodimer abundance, and so we examined their relationship to each other by simulating the effect of IκBβ deficiency in the context of p50-containing or p50-deficient cells. In p50-deficient cells, compensation by p52 is observed but remains incomplete; in our simulations we account for that with a 30-fold reduction in p50 synthesis rates (qualitatively similar results were obtained with a range of synthesis rates; Fig. 6a). We find that loss of IκBβ in p50-deficient cells result in lower A:A abundance, although the A:A homodimer remains detectable in contrast to results in p50-containing cells, in which A:A abundance was more IκB-dependent (Fig. 5f,g).

To test the validity of this model prediction, we generated p50/IκBβ-doubly-deficient MEFs and compared relative abundances of RelA homodimers as determined using electrophoretic mobility shift assays (EMSA; Figs 1c and 6b). We found that in good agreement with the model predictions, IκB deficiency not in IκBα-deficient cells (Fig. 5f). Improbable parameter affinity sets yielded variable amounts of A:A homodimer abundance in the three genotypes. Our experimental analysis supported the probable affinity sets’ predictions: IκBβ-deficient cells showed a dramatic drop (<2% of wild type) in the A:A homodimer abundance as revealed by deoxycholate treatment of cytoplasmic extracts, whereas IκBα deficiency did not appear to alter the A:A homodimer repertoire (>97% of wild type; Fig. 5g). We then determined which of the parameter sets passed the above three tests. Remarkably, none of the improbable parameters allowed for correct predictions, while two parameter sets from the initially determined probable range did (Supplementary Table 5). These three model-directed experimental studies thus support the notion that a high affinity for the A:A homodimer endows IκBβ with a stabilizing role in the abundance of the A:A homodimer. Interestingly, two NFκB target genes (MIP-2 and TNFα), whose expression was found to be mediated by RelA:RelA homodimers, showed a marked reduction in IκBβ-deficient cells (particularly at late time points), whereas expression of RelA:p50 target genes was not reduced but elevated instead (Supplementary Fig. 3).
Figure 5 | Iterative testing and refinement of the IkBβ-RelA homodimer preference model. (a) A model schematic of the Rel-NFκB dimer generation module, as in Fig. 3a but with two IkBs, IkBα and IkBβ, necessitating i₁ and i₂ synthesis rate constants, i₁₋ and i₁₋ degradation rate constants, d₁₋d₄ and d₁₋d₄ IkB-NFκB association rate constants. d₁₋, d₁₋, d₁₋ and d₁₋ IkB-NFκB dissociation rate constants, and d₁₋ d₁₋ and d₁₋ d₁₋ IkB-NFκB degradation rate constants of IkB to release NFκB. (b) Computational predictions of RelA protein abundance in A:50-deficient cells (relative to wild-type cells), using probable (red) and improbable (blue) affinities shown in Fig. 4b and listed in Supplementary Table 5. (c) Immunoblot against RelA of whole-cell extracts from RelA-deficient, wild type and RelA-only MEFs. The right panel shows the quantitation of three independent experiments. These consistently showed values of >0.5. (d) Computational predictions of the abundances of NFκB dimers RelA-RelA (left) and RelA:p50 (right) bound to either IkBα or IkBβ as indicated. These calculations used probable (red) and improbable affinities (blue) as in b. (e) EMSA of DOC-treated cytoplasmic extracts (which results in the separation of NFκB dimers from IkBs) to quantitate IkB-bound NFκB dimer abundance. Prior immunodepletion of the extracts with antibodies against IkBα or IkBβ allows quantitation of NFκB bound to the remaining IkB isofrom. Data shown are representative of three independent experiments, and the right panel shows the corresponding quantitation. (f) Computational predictions of the basal abundances of the RelA:RelA homodimer in wild-type, IkBβ-deficient and IkBα-deficient MEFs using probable (red) and improbable (blue) affinities as in b. (g) EMSAs (left panel) of DOC-treated cytoplasmic extracts of wild-type, IkBβ-deficient and IkBα/c-deficient MEFs. Shown is a representative result of three independent experiments, and the right panel shows the corresponding quantitation.
Figure 6 | IκBβ is important in RelA homodimer formation. (a) Bar graph depicting the simulation results of NFκB dimer abundances in wild-type, IκBβ-deficient, p50-deficient and IκBβ/p50 doubly deficient MEFs. Compensation by p52 in p50-deficient cells was simulated for antibody synthesis of a p50-like molecular species. (b) EMSA of nuclear extracts prepared from p50-deficient and p50/IκBβ-deficient MEFs treated with TNF at 10 and 30 min. In the presence of p50-mediated monomer competition, the RelA:RelA homodimer is highly abundant, even in the absence of IκBβ. Data shown are representative of three independent experiments.

Discussion

The described work reveals a novel and specific function for IκBβ. IκBβs are named 'inhibitors of NFκB' because they are capable of inhibiting NFκB DNA-binding activity in cell-free in vitro assays3,9, and IκBβ was identified in that same manner22. However, we show here that in vivo IκBβ functions primarily as a positive regulator of NFκB dimer generation. Whereas the RelA:p50 dimers can readily form, owing to a high dimerization affinity, RelA:RelA homodimer formation requires the stabilizing influence of IκBβ. These distinct functions of IκBβ and other IκBs explain the phenotypes observed in knockout mice.

Previous studies of the IκB–NFκB signalling module revealed that negative feedback by IκBα, IκBβ, and IκBδ shape the dynamics of NFκB activity in response to stimuli12,20,21,23–25. Indeed, prolonged NFκB activity leads to a severe hyperinflammatory phenotype in IκBβ-deficient mice, and to hyperpolarization in B cells lacking IκBε (ref. 26) or IκBδ (ref. 36). However, IκBβ-deficient mice were recently reported to have hypoinflammatory responses, reduced expression of TNF and resistant to endotoxic shock and collagen-induced arthritis27. We show here that IκBβ-deficient fibroblasts lack one member of the NFκB dimer family, namely the homodimer of the potent activator RelA (Fig. 3). Similar resultant RelA:RelA and RelAcRel deficiency has been observed in iκbβ−/− macrophages5.

Previously established mathematical models of the IκB–NFκB signalling module are unable to account for this phenotype. These models assume a constant amount of NFκB dimer as a given quantity, and calculate the control of its subcellular localization and DNA-binding activity as a function of dynamically regulated NFκB degradation and re-synthesis. Here we have constructed a model for the Rel-NFκB dimer generation module and parameterized based on biophysical measurements of recombinant proteins and a series of iterative cycles of prediction and in vivo measurements. Our work reveals that IκBβ is critical for NFκB RelA:RelA homodimers in MEFs, whose spontaneous generation is challenged not only by poor intrinsic dimer affinities, but also by competition with the high-affinity heterodimerization partner p50.

A key feature of IκBβ function is an unusually high affinity for RelA:RelA homodimers (3.2 pM). As recombinant IκBβ is not monodispersed in cell-free systems, this model-aided interpretation of cellular measurements cannot be tested directly. While subnanomolar dissociation constants have previously been reported for other protein–protein interactions28, it may be prudent to reconsider the simplifying assumption of the coarsegrained model formulation, in which IκBβ interacts only with the pre-formed dimer. Instead, IκBβ might be able to bind monomers sequentially (Supplementary Fig. 5a), in which case IκBβ may be considered a bona fide chaperone for RelA:RelA

These two roles as a function of the affinity between IκBβ and the A:A homodimer. We simulated both the basal abundance of the A:A homodimer and the duration of A:A homodimer activity above the threshold of a 50% of maximum activity (Supplementary Fig. 4c) as a function IκBβ:A:A homodimer affinities over a thousandfold range (Fig. 7d). We found that lowering the IκBβ:A:A affinity would prolong A:A homodimer activity following transient stimulation, but have little impact on A:A abundance; however, increasing the affinity would increase the basal abundance of the A:A homodimer. We thus conclude that the IκBβ affinity for the A:A homodimer is tuned to be low enough that IκBβ does not function within the Rel-NFκB dimer generation module, but high enough so that it functions within the IκB–NFκB signalling module to control the dynamics of A:A activity.
homodimer formation. Whereas the single-step reaction scheme in the original model is $Kd = \frac{[A][B]}{[AB]}$, the two-step reaction scheme would be $Kd = \frac{[A][B]}{[AB]+[A][B]}$. Resistance to negative feedback may be imparted particularly to those NFKB dimers (for example, A:A and A:B) that show high affinity for IκBα (refs 5,27). As such, both the proposed nuclear function and the here-described chaperone function are a consequence of the high affinity for specific dimers revealed through our iterative experimental and computational study.

The parameterization of computational models is a recognized challenge of systems biology. Whereas strategies have been developed to parameterize mathematical synthesis and degradation expressions, as well as those describing enzymatic functions within cells, the interactions between proteins are rarely quantitatively addressed. Ex vivo, affinity and kinetic rate constants can be determined for the interaction of recombinant proteins; however, the in vivo relevance of such constants is limited by the difficulties of producing recombinant full-length proteins with appropriate post-translational modifications. Here we exploited the fact the IκB abundance is a function of its interaction with NFKB (ref. 12; Fig. 2b) and employed computational modelling in order to derive in vivo interaction affinities from immunoblot quantifications. Interestingly, it was previously observed that RelA deficiency results in IκBα deficiency, and such obligate interactions are not rare, suggesting that our model parameterization strategy may be employed for other biological systems.

We described the Rel-NFKB dimer generation module and the IκB–NFKB signalling module as distinct but there are of course numerous interactions. At a minimum, we may consider that IκBα, within the Rel-NFKB dimer generation module, functions as a ‘licensing factor’ for subsequent dynamic control by IκBβ. The distinct functions by these structurally homologous proteins are on the basis of both their specific protein-interaction constants, and on their distinct dynamic degradation and synthesis control. Indeed, the regulatory utility of such ‘hand-off’ or ‘relay control’ network motifs, based on kinetic differences between family members, may be a driver of the expansion of protein families.
isomers may affect the abundance of these dimers; model 4 addresses how nuclear localization of these NFкB dimers is induced and regulated following TNF-induced IKK activation.

Model 1 (Fig. 1a, Supplementary Note 1) contains RelA and p50, which form three NFкB dimers: RelA:p50 and p50:p50. The rate constants for the parameters in these equations are found in Supplementary Table 1.

Model 2 (Fig. 3a, Supplementary Note 2) is based on model 1 above. A generic IкB can complex with RelA and RelA:p50. This IкB has not been demonstrated to bind to IкB, so that interaction is excluded from this model. We assume no degradation of the IкB–NFкB complex or the NFкB dimer from the complex; the dimer may dissociate or IкB may degrade and release the NFкB dimer. The rate constants for the parameters in these equations are found in Supplementary Table 2.

Model 3 (Fig. 5a, Supplementary Note 3) is based on Model 2. IкBz/c and IкBx are the specific IкBs in the model, which can complex with RelA:A and RelA:p50. For simplicity, IкBx and IкBz are assumed to have the same molecular interaction characteristics and are treated as a single IкB species. The rate constants for the parameters in these equations are found in Supplementary Table 3.

Parameterization of the model required exploration of the parameter space for IкB–NFкB affinities and determining the likelihood of each set of parameter values (see results Fig. 4b). Further parameterization involved additional experimental data (Fig. 5) in order to eliminate nonconforming parameter sets (see Supplementary Table 5).

Deriving IкB–NFкB affinities. Model 2 was used to calculate the IкB abundances in RelA:p50-deficient and RelA:RelA-deficient cells using NFкB dimerization affinities (determined by 31 sets of d parameters for homodimers and 31 sets of heterodimers) and IкB–NFкB interaction affinities (determined by 61 sets of di parameters for IкBz and 61 for IкBx). NFкB dimerization affinities were measured with an increased sensitivity of twofold, and so each IкB abundance was assumed to have the same molecular interaction characteristics and treat them as a single IкB species. The rate constants for the parameters in these equations are found in Supplementary Table 3. The model ODEs were solved numerically using MATLAB version R2013a (The MathWorks Inc.). A subroutine ode15s was used. Relaxation of 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 4,000 min. Stimulus-induced perturbation from the steady state of Model 4 was accomplished by direct modulation of IKK activity via a numerical input curve defined as showing no concentration changes greater than 1% over a period of 30 min.

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Protection Assay23 and immunoblotting21 were performed as previously described. Briefly, cells were grown to confluence in Dulbecco’s Modified Eagle’s Medium supplemented with 10% bovine calf serum, 1% penicillin-streptomycin and 1% L-Glutamine. Stimulations were performed using 1 ng/ml TNF (Roche). The cell lines used were as follows: wild-type (ref. 21), IкBβ−/− (ref. 23), IкBα−/− (ref. 21), IкBα−/−/IкBβ−/− (ref. 12), rela−/− (ref. 7), relb−/− (ref. 12), relb−/−/IкBβ−/− (ref. 7), relb−/−/ relb−/relb− (ref. 12), and relb−/relb−/IкBβ−/−/IкBβ−/− (ref. 7). Akb−/−/IкBα−/−/IкBβ−/− knockout mice were generated from E12.5–14.5 embryos and immortalized following the 3T3 protocol.

Cell culture. Immortalized MEFs were prepared and cultured as previously described. Briefly, cells were grown to confluence in Dulbecco’s Modified Eagle’s Medium supplemented with 10% bovine calf serum, 1% penicillin-streptomycin and 1% L-Glutamine. Stimulations were performed using 1 ng/ml TNF (Roche). The cell lines used were as follows: wild-type (ref. 21), IкBβ−/− (ref. 23), IкBα−/− (ref. 21), IкBα−/−/IкBβ−/− (ref. 12), rela−/− (ref. 7), relb−/− (ref. 12), relb−/−/IкBβ−/− (ref. 7), relb−/−/ relb−/relb− (ref. 12), and relb−/relb−/IкBβ−/−/IкBβ−/− (ref. 7). Akb−/−/IкBα−/−/IкBβ−/− knockout mice were generated from E12.5–14.5 embryos and immortalized following the 3T3 protocol.

Biochemistry. EMSA21, deoxycholate (DOC) + EMSA24, EMSA supershift33, wild-type + mutant oligo competition EMSA, immunodepletion, RNase Protection Assay22 and immunoblotting22 were performed as previously described. Briefly, for EMSA, nuclei were collected by hypotonic lysis and resuspended in 30 μl NE buffer (250 mM Tris pH 7.8, 60 mM KCl, 1 mM EDTA, with freshly added 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF) and lyzed by three freeze-thaw cycles. Nuclear lysates were cleared by 14,000g centrifugation and adjusted to 1 μg/ml total protein concentration. Total nuclear protein (2 μg) was incubated at room temperature for 15 min with 0.1 μmol of P2 probe labeled by 32P by double-stranded oligonucleotide containing a consensus κB site (5′-AGCCTGCTCAAGGGACTTTCCGCGTGCTCACTATT-3′) in 3 μl binding buffer (10 mM Tris-Cl pH 7.5, 50 mM NaCl, 10% glycerol, 1% NP-40, 1 mM EDTA, 0.1 μg/ml poly(dI:dC)). Complexes were resolved on a non-denaturing gel and visualized using a Typhoon Scanner (GE Healthcare Life Sciences). For DOC sensitivity, 2 μg of nuclear extract was treated with DOC to a final concentration of 30% for 30 min and subsequently subjected to EMSA analysis. For supershifts, nuclear extracts were incubated with indicated antibody cocktails for 20 min on ice before probe incubation followed by resolution on non-denaturing gel. For wt-mutant competition EMSA, nuclear extracts were incubated with 100-fold molar excess of wt and mutant κB probes and resolved as before. For RNase Protection with total RNA and cDNAs was made and subjected to EMSA analysis. For supershifts, nuclear extracts were incubated with indicated antibody cocktails for 20 min on ice before probe incubation followed by resolution on non-denaturing gel. For wt-mutant competition EMSA, nuclear extracts were incubated with 100-fold molar excess of wt and mutant κB probes and resolved as before. For RNase Protection with total RNA and cDNAs was made and subjected to EMSA analysis. For supershifts, nuclear extracts were incubated with indicated antibody cocktails for 20 min on ice before probe incubation followed by resolution on non-denaturing gel. For wt-mutant competition EMSA, nuclear extracts were incubated with 100-fold molar excess of wt and mutant κB probes and resolved as before. For RNase Protection with total RNA and cDNAs was made and subjected to EMSA analysis.
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Author contributions

Computational modelling work was performed by R.T. and J.D.K., and experimental work by C.L., K.N., S.B., R.T., D.V., K.N. and J.D.K., with supervision from A.H. and G.G. R.T. and A.H. wrote the manuscript, based on an early draft by J.D.K. A.H. planned and oversaw the project.

Additional information

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