Substrate complex competition is a regulatory motif that allows NFκB RelA to license but not amplify NFκB RelB

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Signaling pathways often share molecular components, tying the activity of one pathway to the functioning of another. In the NFκB signaling system, distinct kinases mediate inflammatory and developmental signaling via RelA and RelB, respectively. Although the substrates of the developmental, so-called noncanonical, pathway are induced by inflammatory/canonical signaling, crosstalk is limited. Through dynamical systems modeling, we identified the underlying regulatory mechanism. We found that as the substrate of the noncanonical kinase NIK, the nfkb2 gene product p100, transitions from a monomer to a multimeric complex, it may compete with and inhibit p100 processing to the active p52. Although multimeric complexes of p100 (IκBδ) are known to inhibit preexisting RelA:p50 through sequestration, here we report that p100 complexes can inhibit the enzymatic formation of RelB:p52. We show that the dose–response systems properties of this complex substrate competition motif are poorly accounted for by standard Michaelis–Menten kinetics, but require more detailed mass action formulations. In sum, although tonic inflammatory signaling is required for adequate expression of the noncanonical pathway precursors, the substrate complex competition motif identified here can prevent amplification of the active RelB:p52 dimer in elevated inflammatory conditions to ensure reliable RelB-dependent developmental signaling independent of inflammatory context.

NFκB canonical pathway | NFκB noncanonical pathway | inflammation | crosstalk | immune development

Signaling pathways are typically thought of as self-contained, receiving a stimulus from a receptor and producing a downstream effect. However, their molecular components often participate in multiple pathways, resulting in highly networked signaling systems (1–3). Interconnected signaling pathways have the potential for stimuli of one pathway to alter the dose–response of another pathway, commonly referred to as signaling crosstalk. Crosstalk has been identified as an important mechanism by which robust regulation is maintained (4), or signals are amplified or fine-tuned (5), but also as a potential route by which defined perturbations have broad effects that may lead to cancer (6, 7). Therefore, defining the mechanisms that insulate from potential crosstalk is of key importance to understanding regulation and misregulation of signaling in health and disease.

NFκB signaling is induced by a wide variety of signals that are transduced by two kinases: the canonical pathway is mediated by NFκB Essential Modulator (NEMO)-containing IKK, and the noncanonical pathway is mediated by NFκB Inducible Kinase (NIK) (8). The canonical pathway is rapidly induced by a variety of inflammatory cytokines and pathogens and through NEMO-dependent degradation of NFκB inhibitors (IκBα/β/ε), which results in nuclear localization of preexisting, transcriptionally active NFκB dimers (predominantly RelA:p50). The noncanonical NFκB pathway leads to the de novo generation of RelB:p52, which controls the developmental maturation of immune cells and organs (9, 10). Developmental and survival stimuli (mediated by LTβR, CD40, BAFFR, RANK, TNFR2, CD27) activate NIK-dependent p100 processing to produce p52, which dimerizes with RelB to produce transcriptionally active RelB:p52. Disruption of RelB:p52 formation, through perturbing p100, RelB, or NIK, affects peripheral lymphoid organ development, dendritic and B-cell maturation, mammary gland development, and osteoclast maturation (11–15). Conversely, constitutive hyperactivity of the noncanonical pathway is associated with broad inflammatory, autoimmune, and malignant pathologies (16, 17).

Whereas the prevailing role of the noncanonical NFκB pathway is in controlling development, organogenesis, and tissue homeostasis, the canonical NFκB pathway functions in an acute, transient manner triggered by pathogen or inflammatory cytokine exposure (16). However, the two pathways are strongly interconnected, as the noncanonical pathway may amplify canonical activation in B cells (18) and in dendritic cells (19). Conversely, canonical pathway activity controls the expression of the two genes that encode the RelB:p52 dimer (i.e., *relb* and *nfkb2*) (20). Indeed, genetic knockouts of canonical pathway mediators such as NEMO, IKK2, or RelA abrogate the ability of the noncanonical pathway to produce RelB:p52 activity (21, 22). Thus, there is potential for cross-regulation that may result in

Significance

Inflammation-responsive canonical NFκB induces many genes, two of which encode noncanonical NFκB pathway components that control developmental processes. This suggests potentially perilous cross-regulation by which inflammatory conditions could derail immune organ developmental decisions. We use mathematical modeling to propose a mechanism that functions as a brake on this connection. We report that the key enzyme mediating developmental NFκB is subject to competition from two forms of its single substrate. Termed substrate complex competition, this regulatory motif can lead to a counterintuitive decrease of signaling product in conditions of elevated substrate abundance. We propose that although noncanonical NFκB requires intact inflammatory NFκB signaling, substrate complex competition allows developmental signals to be reliably transduced without inappropriate amplification by inflammation.

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substantially elevated noncanonical NFκB (RelB:p52) activity in elevated inflammatory conditions that would derail normal tissue homeostasis. Fortunately, this is not the case: the debilitating phenotypes observed in hyperactive noncanonical pathway mutants (17, 23) are not observed in inflammatory conditions or in constitutively active mutants of canonical pathway components (24). This suggests that there is a regulatory brake that limits the extent by which canonical pathway can modulate noncanonical pathway activity. However, the mechanistic basis of this brake remains unknown.

Here we investigate how insulation between canonical and noncanonical pathway activity is achieved to ensure that inflammatory signals do not adversely affect developmental homeostasis of immune cells and organs. Using dynamical systems modeling, we discover and characterize a regulatory motif in which the critical signal-transducing kinase is presented with alternate complexes of the substrate, resulting in substrate complex competition. This, in turn, modulates the enzymatic substrate-to-product dose–response in a manner that may result in an effective insulation of one pathway from the other.

Results

A Computational Model of NFκB Predicts Amplification of Noncanonical RelB:p52 by Canonical Pathway Activity. To investigate crosstalk between the canonical and noncanonical NFκB pathways, an established mathematical model of NFκB signaling (25) was extended to include canonical pathway–induced p100 and RelB synthesis, along with NIK-dependent p100 to p52 processing (Fig. 1*A*). This network topology has potential for the canonical (inflammatory) pathway to amplify noncanonical (developmental) signals mediated by RelB:p52. Indeed, computational simulations of this network predicted that canonical pathway induction (reflected in p100 mRNA levels) substantially induces not only the canonical RelA:p50 dimer but also the noncanonical RelB:p52 dimer (Fig. 1*B*).

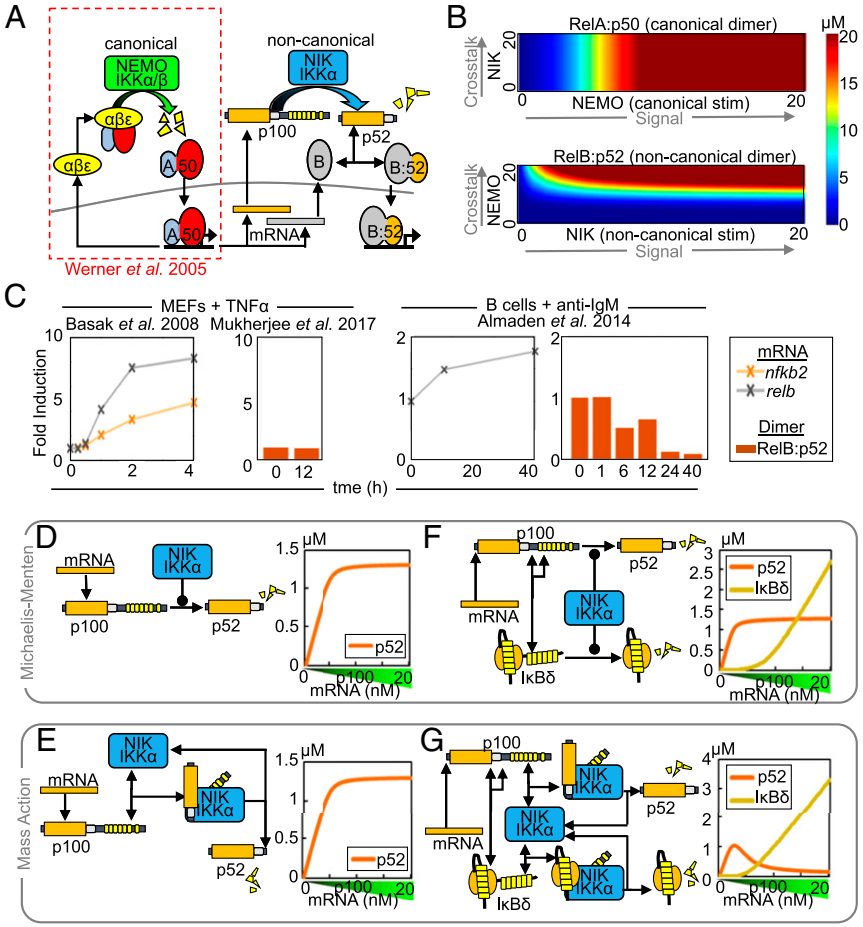

Fig. 1. Substrate complex competition results in reduced product formation with increasing substrate. (*A*) Schematic of the computational model adapted from Werner et al. (25) with the addition of NFκB-responsive synthesis of p100 and RelB, NIK-mediated processing of p100 to p52, and subsequent p52 binding to RelB. Each reaction (represented by an arrow) is represented by an ODE. In the absence of stimulation, canonical NFκB RelA:p50 is predominantly bound to IκBs. In response to canonical stimuli, IκBα/β/ε are degraded in a NEMO-dependent manner, releasing NFκB RelA:p50 to the nucleus, where it activates transcription of IκBs, forming a feedback loop, as well as p100 and RelB. p100 is processed into p52 in a NIK-dependent manner in response to noncanonical stimuli, which can then bind RelB to form RelB:p52 and translocate to the nucleus. (*B*) Heat maps of simulated steady-state concentrations of nuclear RelA:p50 and RelB:p52 as a function of canonical and noncanonical signaling strengths. (*C*) Experimentally determined timecourse line graphs of *relb* and *nfkb2* (encodes p100 protein) mRNA induction in MEFs (*Left*) and B cells (*Right*) in response to canonical-pathway-activating stimuli TNFα (*Left*) and anti-IgM (*Right*). MEF data are quantified from Basak et al. (22), B-cell data from Almaden et al. (18). Bar graphs show RelB:p52 induction quantified by Mukherjee et al. (26) and Almaden et al. (18). (*D* and *E*) Schematic (*Left*) and steady-state concentration of p52 (*Right*) as a function of p100 mRNA concentrations as predicted by computational models of NIK-mediated p100 processing based on Michaelis–Menten kinetics (*D*) or mass action kinetics (*E*). (*F* and *G*) Schematic (*Left*) and steady-state concentration of p52 and IκBδ (*Right*) as a function of p100 mRNA concentrations as predicted by computational models of NIK-mediated p100 processing and IκBδ degradation based on Michaelis–Menten kinetics (*F*) or mass action kinetics (*G*). IκBδ is modeled as p100 dimer (double-headed arrow). The substrate complex competition motif is described by *G*.

Although canonical pathway activity does indeed induce both p100 (*nfkb2*) and RelB mRNA in mouse embryonic fibroblasts (MEFs) (22) (Fig. 1*C*), amplification of the transcriptionally active RelB:p52 protein dimer, as predicted by the computational simulation, is not observed experimentally [Fig. 1*C* (26)]. Similarly, in B cells, *nfkb2* mRNA is induced by canonical pathway activity, but amplification of RelB:p52 is not seen (18). Indeed, such amplification would be deleterious to developmental processes in the context of chronic inflammation (18, 22, 26). To establish why the computationally identified potential for substantial crosstalk is not realized, and examine the discrepancy between mRNA induction and protein dimer formation, mechanistically more detailed mathematical models of NIK-mediated reactions were constructed (Fig. 1 *D* and *E*).

The dose–response of the noncanonical NFκB monomer p52 to increasing canonical pathway activity was characterized: a typically saturating dose–response curve of p52 to increasing p100 mRNA was observed whether p100 processing was represented by a Michaelis–Menten equation (Fig. 1*D*) or mass-action kinetics of two-step enzymatic binding and processing reactions (Fig. 1*E*).

Competition Between Distinct Forms of NIK's Substrate Can Lead to an Inverted Dose–Response Regime. p100, if not processed into p52, forms higher-molecular-weight NFκB-inhibitory complexes, also known as IκBδ-containing IκBsomes (27–29). It was shown that, on p100 homodimerization via the rel homology domain, one monomer's ankyrin repeat domain self-inhibits the dimer, and the other remains available to inhibit an NFκB dimer in *trans*. Although newly synthesized monomeric p100 can be processed into p52 by NIK, the exposed ankyrin repeat domain of the p100 homodimer (IκBδ) is degraded in a NIK-dependent manner, leaving only a self-inhibited nonfunctional p100:p52 heterodimer. We represent p100:p100 processing to nonfunctional p100:p52 as complete degradation of IκBδ, consistent with previous mathematical models (30, 31). Simulations with a model of NIK-mediated p100 processing into p52 based on Michaelis–Menten kinetics predicted no change to the dose–response of p52 with the addition of NIK's role in the degradation of IκBδ (Fig. 1*F*). However, when these reactions were modeled using a two-step formulation with mass-action kinetics (binding and catalysis by the enzyme), p52 first reaches a lower maximal level and then shows an inverted dose–response relationship with increasing substrate (p100 mRNA), resulting in decreased product (p52; Fig. 1*G*). At the stationary point of maximal p52, either increasing or decreasing canonical signaling strength will reduce p52 abundance. Formulating the reactions with detailed mass action kinetics revealed that the free NIK concentrations available for processing one substrate complex can be affected by its processing of the other; specifically, NIK binding to multimeric p100 (IκBδ) excludes it from binding newly synthesized monomeric p100. We refer to this process as substrate complex competition. Although single-substrate dose–response relationships were indistinguishable in Michaelis–Menten models of a single enzyme-mediated reaction and two-step mass-action representations, they differed when two substrate complexes were considered, regardless of the values in p100 dimerization kinetics, NIK enzymatic activities, and IκBδ degradation rates (*SI Appendix*, Fig. S1). The nonmonotonic dose–response of substrate complex competition is also observed when degradation of NIK after enzymatic activity is included to capture the possibility of a negative feedback loop by which IKKα that has been activated by NIK can in turn phosphorylate and destabilize NIK [*SI Appendix*, Figs. S2 and S3 (32)].

NIK Abundance Determines Whether Substrate Complex Competition Occurs. To probe the mechanism further, we examined the control of the stationary point on the p52 dose–response curve with parameter scans of p100 mRNA (p100t, which we used as an indicator of canonical pathway/NEMO activity) over a wide variety of NIK concentrations (Fig. 2*A*). Expectedly, in a regime lacking NIK, p52 was not produced at any level of p100 mRNA, as p100 processing is NIK-dependent. At a wide range of intermediate NIK levels, initial increases of p100 mRNA resulted in increasing p52 as NIK binds to nascent p100 (Fig. 2 *A* and *B*). After the initial increase in p52, as NIK begins to reach saturation, more unprocessed p100 forms IκBδ, resulting in NIK–IκBδ complexes, which compete with nascent p100 for NIK binding (Fig. 2*B*). This substrate complex competition results in a decrease in p52 formation. In a regime with excess NIK [>16-fold higher than published parameters (18)], NIK does not reach saturation, enabling complete processing of all nascent p100 into p52, and no IκBδ is predicted to form with increasing canonical pathway activity (Fig. 2*A*). Thus, the excess NIK regime is predicted to show monotonic crosstalk as all increases in canonical pathway activity result in increased p52 production. However, for a wide range of NIK abundances [around the published kinetic parameters (18)], biphasic crosstalk is predicted with the canonical pathway, boosting p52 production at the low end and diminishing p52 production at the high end (Fig. 2*A*).

Interestingly, for B cells cultured in B cell activating factor (BAFF), published kinetic parameters predict that canonical pathway activity is approximately optimal for peak p52 production; both decreases and increases in canonical pathway activity would lead to reduced p52 generation resulting from either reduced substrate availability or substrate complex competition (Fig. 2 *A* and *B*). However, when NIK abundances are further elevated, higher p100 mRNA levels are required to saturate NIK, and higher maximal NIK-p100 abundances are obtained (Fig. 2*C*). In the 16-fold range of NIK abundances around the published parameters, there is a fundamental limit on NIK-p100 complex formation resulting from substrate complex competition, which limits crosstalk and ensures that p52 can only be substantially induced by increasing NIK concentration through the noncanonical pathway.

Although the regime of NIK activity determined by previously published parameters (18) indicates a substantial role of substrate complex competition, we sought to generate an experimentally testable prediction to further explore its biological relevance. Through a timecourse simulation of increasing p100 mRNA (resulting from canonical NFκB pathway activity), we predicted that substrate competition leads to NIK switching substrates from nascent monomeric p100 to IκBδ, resulting in a decrease in p52 generation coinciding with an increase in p100 (mainly in the multimeric p100 form of IκBδ; Fig. 2*D* and *SI Appendix*, Fig. S4). Indeed, when B cells cultured in the presence of the noncanonical stimulus BAFF are stimulated with canonical NFκB pathway stimulus anti-IgM, the substantial constitutive levels of p52 then decrease between 8 and 25 h, whereas p100 increases (Fig. 2*E*) (18). This timecourse closely matches the one predicted by the computational model. The mechanism for this p52 decrease is NIK switching from predominantly binding to nascent p100 (which results in p52 production) to predominantly binding to IκBδ (Fig. 2*D*). We conclude that in B cells, NIK activity was indeed in the concentration regime predicted by published parameters in which NIK becomes substrate saturated when cells are stimulated by canonical stimuli. Therefore, we identified substrate complex competition as a potential mechanism of reducing noncanonical pathway activity in response to canonical signals.

Substrate Complex Competition Can Insulate RelB:p52 from Canonical Pathway Crosstalk by Reversing the Dose–Response of p52. Although the reduced model allowed us to investigate the potential for a biphasic dose–response curve resulting from substrate complex competition, we next investigated the effect of this proposed

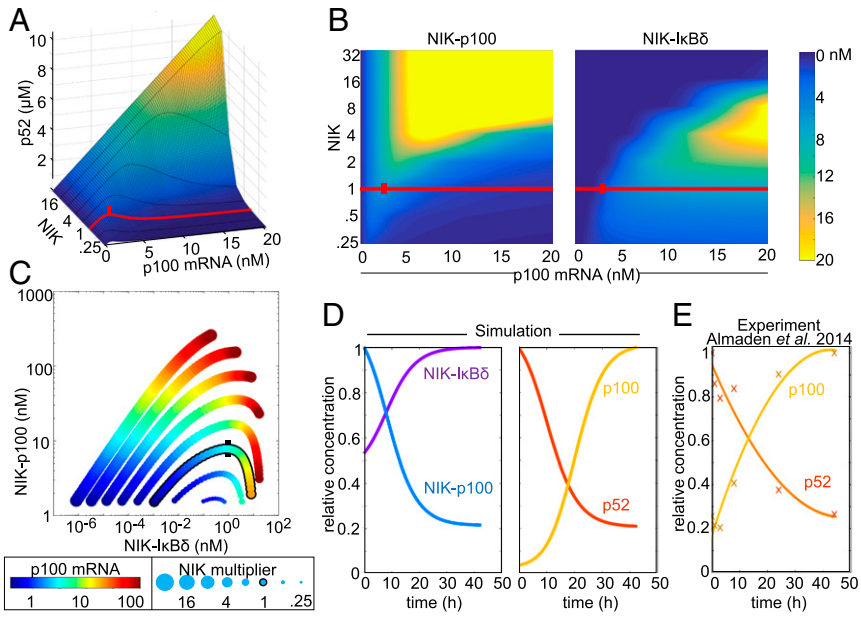

Fig. 2. Canonical NFκB signaling reduces p52 as a result of substrate complex competition. (*A*) 3D surface plot of simulated steady-state concentration of p52 as a function of increasing p100 mRNA concentrations and relative NIK levels using the mass action model depicted in Fig. 1*G*. A red line indicates reported basal levels of NIK activity, with the reported basal p100 mRNA level indicated with a thick mark (18). Here, p100 mRNA is an indicator of canonical NFκB RelA:p50 activity. (*B*) Heat maps of the simulated concentrations of NIK-p100 and NIK-IκBδ complexes, generated as in *A*. (*C*) Scatter plot showing the relationship between the concentrations of NIK-IκBδ and NIK-p100 complexes for different levels of NIK, as p100 mRNA levels were increased (color scale). Shown are values from the first point in a timecourse simulation when p52 exceeds 200 nM. The full parameter range was scanned for every NIK concentration, but only simulations in which p52 exceeded 200 nM are shown. Basal NIK activity is indicated with a black border with the thick mark indicating the basal p100 mRNA concentration. (*D*) Line plots of simulated timecourse concentrations of NIK-p100 and NIK-IκBδ complexes (*Left*) and p100 and p52 proteins (*Right*) in response to canonical pathway activity using the p100 mRNA input curve shown in *SI Appendix*, Fig. S3. Concentrations were normalized to their maximum value. p100 was plotted as the sum of all molecular species containing p100 for consistency with experimental assays (p100, NIK-p100, IκBδ, and NIK-IκBδ). (*E*) Line graphs from quantified Immunoblots of whole-cell p52 and p100 expression in wild-type B cells stimulated with anti-IgM reported by Almaden et al. (18).

mechanism on the formation of the transcriptionally active NFκB dimer RelB:p52. Similar to p100, RelB is a target gene of canonical NFκB (RelA:p50) activity (Fig. 1*C*), but unlike p52, it does not require processing. The working model was therefore extended to include inducible expression of RelB to investigate how the nonmonotonic dose–response of p52 (resulting from substrate complex competition) and the monotonic dose–response of RelB combine to control RelB:p52 activity (Fig. 3*A*).

As expected, simulations showed that increasing NEMO kinase activity led to increases of p52 at the low range, but decreases in the high range (Fig. 3*B*), whereas RelB levels increased substantially, as seen in multiple experimental systems (Fig. 1*C*). An elevated level of NIK activity could shift the saturation point to allow for more p52 generation. Interestingly, the combined result of RelB and p52 responses to increasing canonical pathway activity is that although RelB is strongly induced, the decreasing availability of p52 resulting from substrate complex competition does not result in hyperactivation of RelB:p52, which remains largely unchanged compared with the magnitude of RelB:p52 induction effected by noncanonical pathway activation (Figs. 1*C* and 3*B*). Only severe deficiency in canonical pathway activity was predicted to substantially diminish RelB:p52 activity because of a lack of both monomers.

To test whether this emergent property accurately reflects experimentally measured responses to canonical and noncanonical stimuli, we scanned both pathway activation strengths from absence to normal basal, to ∼fivefold over basal (Fig. 3*C*). We found that induction of NIK is expected to strongly induce RelB:p52, but interestingly, the model predicted that combining NIK activation with activation of the canonical kinase NEMO would further induce RelB, but not the RelB:p52 dimer, because of substrate complex competition limiting the generation of p52. This finding is robust to the fold change in half-life that occurs when monomeric p100 and p52 dimerize into a more stable RelB:p52 heterodimer (*SI Appendix*, Fig. S5). Indeed, comparing this prediction to experimentally measured nuclear RelB:p52 in B cells stimulated with NIK-activating stimuli BAFF and costimulation with the addition of NEMO-activating stimuli anti-IgM confirmed no amplification of RelB:p52 by coactivation of the canonical pathway activity [Fig. 3*D* quantified from published data (18)], compared with Fig. 3*C* RelB:p52 plot. Remarkably, the slight reduction in RelB:p52 with canonical costimulation predicted by the model is reproduced in these experimental conditions. The model also predicts that RelB:p52 formation is abolished, even in response to NIK activation, if basal canonical pathway activity is removed (Fig. 3*C*). Indeed, this is experimentally confirmed, as in MEFs, genetically deficient in IKKβ (a component of the canonical NEMO IκB-kinase complex), RelB:p52 is diminished and cannot be induced by NIK-activating stimuli (Fig. 3*D*).

Overall, RelB:p52 activity levels are predominantly controlled by NIK with a requirement for a minimal level of canonical signaling to avoid substrate and RelB limitation. We term this requirement for basal NEMO signaling as licensing, as basal canonical signals enable noncanonical signaling. However, elevated canonical activity was unable to further amplify it. Indeed, in the high canonical activity regime, the combination of opposite dose–response curves of p52 and RelB results in an effective insulation of noncanonical RelB:p52 from canonical RelA:p50 transcription factors.

Discussion

Biological signaling pathways consist rarely of linear cascades of enzymes, but rather of a complex networks of enzymes that act on multiple molecular substrates. Given the high potential for

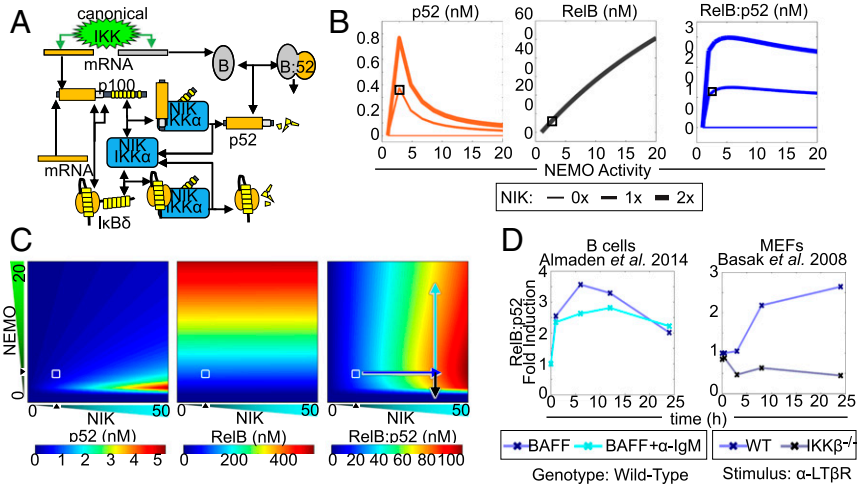

Fig. 3. Transcriptionally active RelB:p52 requires, but is not amplified by, canonical NFκB RelA:p50 activity. (*A*) Schematic representing the extended model of RelB and p100 induction by canonical pathway activity. (*B*) Line graphs of simulated steady-state p52 (*Left*), RelB (*Middle*) and RelB:p52 (*Right*) in response to increasing NEMO activity (represented by increasing p100 mRNA and RelB expression). Dose–responses are shown for simulations with no NIK activity (thin line), basal NIK (moderate line thickness) activity and twofold increased NIK activity over basal (thicker line). Basal NEMO and NIK activities are indicated with a black square. Here, p100 mRNA production is used as an indicator of canonical pathway activity. (*C*) Heat maps of simulated steady-state p52 (*Left*) RelB (*Middle*) and RelB:p52 (*Right*) over a parameter scan of NIK activity and NEMO activity. Basal NEMO and NIK activities are indicated with a white square. Horizontal arrow indicates increases from basal NIK. Increasing vertical arrow indicates the predicted effect of canonical pathway costimulation. Decreasing vertical arrow indicates the predicted effect of the reduction of basal canonical pathway activity. (*D*, *Left*) Timecourse of RelB:p52 NFκB DNA-binding activities in B cells stimulated with BAFF alone (noncanonical stimulation), and anti-IgM (canonical pathway stimulation) plus BAFF quantified from Almaden et al. (18). (*D*, *Right*) Timecourse of RelB:p52 NFκB DNA-binding activities in WT and IKKβ$^{-/-}$ (canonical pathway kinase knockout) MEFs stimulated with α-LTβR (noncanonical stimulation).

regulatory crosstalk, how systems achieve pathway insulation leading to the robust and predictable responses required to maintain homeostasis and health is an important question.

Here we have proposed a mechanism that substantially modifies the expected dose–response curve between two pathways. The mechanism is termed substrate complex competition, in which an enzyme's substrate has the propensity to form a complex that may also be recognized by the enzyme, but does not lead to the functionally active product. Thus, excess expression of the substrate will lead to buildup of the competing complex, and a reduction in enzymatic flux and product. Although substrate competition, in which catalysis of one substrate inhibits an enzyme's ability to catalyze other substrates has been described (33, 34), the motif described here is distinct, in that a single substrate is capable of forming alternate forms (oligomeric complexes) that lead to functionally distinct products. This feature alone leads to the striking nonmonotonic dose–response relationship described here. A nonmonotonic dose–response relationship has previously been described to require substantially more complex regulatory networks, including expression of additional inhibitors, feedback mechanisms, or multiple phosphorylation states (35).

It was opined that enzymes within signal-transduction pathways may not always satisfy preconditions of Michaelis–Menten kinetics, including the requirement that substrate concentration greatly exceeds enzyme concentration (36). In the case of NIK (also known as MAK3K14), whose specific activity is not regulated (via posttranslational phosphorylation), unlike other MAP3K family members, both enzyme concentration (determined by regulating its degradation) and substrate concentration (determined by canonical pathway activity) are highly variable. We showed that the Michaelis–Menten equation accurately accounts NIK's dose–response without substrate complex formation; however, the Michaelis–Menten formulation fails to recapitulate the effects of substrate complex competition and renders incorrect dose–response relationships when multiple functionally distinct substrate isoforms impinge on the same enzyme. To account for substrate complex competition with a Michaelis–Menten rate equation, each oligomeric complex of the substrate would need to be modeled as a competing substrate (*SI Appendix*, Fig. S6) and cannot be recreated by perturbing parameters within the standard Michaelis–Menten representation (*SI Appendix*, Fig. S1) (34).

Interestingly, marked distinctions between Michaelis–Menten and step-wise representations have been observed in the canonical MAPK signaling cascade (37); however, these resulted in a quantitative difference in dose ranges (rather than the qualitatively reversed dose–response seen here) and resulted from a distinct mechanism (conserved moieties rather than substrate complex competition). Here we highlight that, although a model formulation may be valid for a process in isolation, when combining models [e.g., for generating whole-cell simulations (38)], enzyme behavior could be qualitatively different in the context of multiple converging substrates or substrate complexes.

It is known that multimeric complexes of p100 (termed IκBδ-containing IκBsomes or kappaBsomes) can sequester preexisting NFκB (e.g., RelA:p50 and cRel:p50) in the cytoplasm in a stochiometric manner, similar to IκBα, β, and ε (18, 19, 27, 28), Here we found that, through substrate complex competition, IκBδ may also kinetically compete for NIK and reduce the processing of precursor p100 into p52. This direct enzyme-mediated brake on NFκB RelB:p52 formation may prevent canonical signaling from amplifying noncanonical signaling independent of IκBδ-mediated NFκB sequestration.

Although substrate complex competition alone does not necessarily diminish signaling crosstalk, within the NFκB signaling network it may. The reason is that the genes of both constituents of the noncanonical dimeric RelB:p52 transcription factor are induced by increasing canonical pathway activity; substrate competition reverses the dose–response of one, thus rendering the dimeric combination of the two, the dimer RelB:p52, remarkably independent of canonical pathway activity, unless that activity is substantially abrogated. As such, we propose a mechanism through which the noncanonical pathway is licensed by basal canonical activity, but that additional canonical activity

does not further amplify RelB:p52 (Fig. 3*C*). The result is that cells receiving developmental noncanonical NFκB signals (e.g., LTβ, BAFF, RANKL) can reliably respond regardless of the inflammatory condition. In other words, even chronic inflammatory conditions do not derail the normal developmental programs that rely on noncanonical NFκB activity unless the mechanisms ensuring complex substrate competition are inactivated (23).

Materials and Methods

Initial computational investigations (Fig. 1*B*) were carried out using an established model as described in *SI Appendix*, *Methods* (25). A computational model of only NIK-mediated reactions with mass action and Michaelis–Menten kinetics (Fig. 1 *D*–*G*) was constructed, and analyzed using COPASI (39). Plots were created in MATLAB (The Mathworks Inc.). Model equations and methodology are provided in the *SI Appendix*. Parameters and initial conditions are provided in *SI Appendix*, Tables S1 and S2. Models are available in COPASI and SBML format (https://www.signalingsystems.ucla.edu/models-and-code/nik/), and on BioModels (40) with the following identifiers: Michaelis–Menten p52 processing only (Fig. 1*D*): MODEL1903280001; mass action, p52 processing only (Fig. 1*E*): MODEL1903280002; Michaelis–Menten, p52 and IκBδ processing (Fig. 1*F*): MODEL1904020002; mass action, p52 and IκBδ processing–substrate complex competition motif (Fig. 1*G*): MODEL1904020003; and mass action, p52 and IκBδ processing, with RelB (Fig. 3*B*): MODEL1904030001.

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