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A Fourth IκB Protein within the NF-κB Signaling Module

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SUMMARY

Inflammatory NF-κB/RelA activation is mediated by the three canonical inhibitors, IκBα, -β, and -ε. We report here the characterization of a fourth inhibitor, *nfκb2*/p100, that forms two distinct inhibitory complexes with RelA, one of which mediates developmental NF-κB activation. Our genetic evidence confirms that p100 is required and sufficient as a fourth IκB protein for noncanonical NF-κB signaling downstream of NIK and IKK1. We develop a mathematical model of the four-IκB-containing NF-κB signaling module to account for NF-κB/RelA:p50 activation in response to inflammatory and developmental stimuli and find signaling crosstalk between them that determines gene-expression programs. Further combined computational and experimental studies reveal that mutant cells with altered balances between canonical and noncanonical IκB proteins may exhibit inappropriate inflammatory gene expression in response to developmental signals. Our results have important implications for physiological and pathological scenarios in which inflammatory and developmental signals converge.

INTRODUCTION

The transcription factor NF-κB plays critical roles in diverse physiological processes (Ghosh and Karin, 2002) and numerous human pathologies (Karin et al., 2004). The primary mediator of NF-κB transcriptional activity is a RelA:p50 heterodimer. Mouse knockout studies have confirmed that RelA is responsible for the expression of a large number of genes involved in inflammatory responses as well as in cell proliferation, cell adhesion, and tissue remodeling (Hoffmann and Baltimore, 2006). NF-κB activity is inducible by a diverse range of stimuli (www.nf-kb.org); these include pathogen derived substances and intercellular mediators of inflammation, immune cell maturation, and secondary lymphoid organ development. Understanding the mechanisms that regulate NF-κB activity is critical for developing therapeutic strategies for many human diseases (Ghosh and Karin, 2002; Hoffmann and Baltimore, 2006; Karin et al., 2004).

Induction of NF-κB/RelA activity in response to inflammatory stimuli does not require protein synthesis. Instead, early experiments distinguished between two activation mechanisms: a precursor processing mechanism or regulation by a separate inhibitor protein. The detergent deoxycholate was shown to liberate fully active κB-site DNA binding activity in unstimulated cells, suggesting the existence of a separate inhibitor protein(s), termed IκB (Baeuerle and Baltimore, 1988). Three IκB proteins (IκBα, -β, or -ε) have been identified, which share the bona fide IκB properties of (1) binding NF-κB dimers, thereby inhibiting their DNA binding activity and retaining them in a latent state, and (2) allowing for NF-κB activation by undergoing stimulus-induced proteolysis. The canonical signaling pathway involves the stimulus-responsive phosphorylation of the IκBs by the NEMO and IKK2 containing kinase complex, which tags them for degradation via the ubiquitin-proteasome pathway. Several other IκB-like ankyrin-repeat containing NF-κB binding proteins have been reported to modulate nuclear NF-κB transcriptional activity on a subset of genes (Yamamoto et al., 2004) or, when overexpressed, prevent nuclear localization of NF-κB (Hatada et al., 1992; Inoue et al., 1992; Naumann et al., 1993). However, during inflammatory signaling the dynamic control of NF-κB nucleocytoplasmic localization is mediated by the three classical IκB proteins. A detailed molecular understanding of the biochemical events has allowed for the construction of a mathematical model that recapitulates the experimentally observed signaling behavior in response to inflammatory stimuli (Hoffmann et al., 2002; Kearns et al., 2006; Werner et al., 2005).

In contrast, a second NF-κB activation pathway is thought to regulate the activity of NF-κB/RelB dimers via a precursor processing mechanism. This signaling pathway is activated in response to developmental signals such as those transduced by lymphotoxin β receptor (LTβR) and RANK, which are required for lymph node and osteoclast genesis and homeostasis (Senftleben et al., 2001; Weih and Caamano, 2003), or BAFFR, CD40, and CD27, which regulate B cell survival and proliferation (Ramakrishnan et al., 2004; Zarnegar et al., 2004). The activation mechanism was shown to involve NF-κB-inducing kinase (NIK) and IKK1-dependent phosphorylation of the *nfkb2* gene product p100 (Dejardin et al., 2002), which triggers its proteasomal processing. This partial proteolysis event removes a C-terminal ankyrin repeat domain (ARD) to generate the NF-κB protein p52 (Senftleben et al., 2001). Interestingly, only newly synthesized p100 was shown to undergo processing to generate nuclear p52-containing nuclear NF-κB activities (Mordmuller et al., 2003).

The physiological role of LTβR is to transduce signals from hematopoietically derived lymphoid tissue inducer cells expressing membrane bound lymphotoxin (LTαβ) to mesenchymal or stromal cells to initiate critical steps in lymph node development (Mebius, 2003; Rennert et al., 1998). LTβR stimulation results in both RelA- and RelB-containing NF-κB dimers (Muller and Siebenlist, 2003). The vascular-cell adhesion molecule 1 (VCAM1), a prominent RelA target gene, mediates early steps in lymph node genesis, the formation of lymph node anlagen during embryogenesis. Subsequently, homing of B cells, whose interaction with stromal cells is critical for lymph node and splenic microarchitecture, requires lymphoid chemokines SLC/CCL21 and BLC/CXCL13, whose expression is thought to involve RelB (Bonizzi et al., 2004). The maturation of spleen and lymph nodes and continued influx and organization of lymphocytes in these secondary lymphoid organs during adulthood is also dependent on LTβR signaling and the expression of the RelA target gene MadCAM (Browning et al., 2005). The phenotypes of knockout mice reflect the requirement of both RelA- and RelB-containing NF-κB activities. While RelA deficiency results in a complete absence of lymph nodes in newborn mice indicating an early organogenic defect (Alcamo et al., 2002), RelB appears to be required for their maintenance as RelB-deficient mice exhibit deterioration of nodes following birth (Weih et al., 2001).

Despite the importance of RelA activity in lymph node genesis, the molecular mechanism responsible for LTβR-induced RelA:p50 dimer activation has remained unclear. Both an IκB-dependent (Muller and Siebenlist, 2003) and an IκB-independent (Jiang et al., 2003) activation mechanism have been proposed, and for CD27 signaling the noncanonical signal transducer NIK has been implicated (Ramakrishnan et al., 2004). Here we report the existence of a fourth bona fide IκB protein that mediates RelA:p50 activation in response to noncanonical NF-κB signaling pathways. Reconstruction of the signaling mechanism in a mathematical model allowed us to demonstrate that this mechanism is sufficient to account for the experimental observables and explore signaling crosstalk in cells exposed to diverse NF-κB inducing stimuli.

RESULTS AND DISCUSSION

NF-κB/RelA Activation without IκB Degradation

Current pathway maps of LTβR signaling posit that IKK1-dependent processing of the *nfκb2* gene product p100 to p52 results in the nuclear localization of a RelB:p52 dimer. However, the activation mechanism of RelA-containing dimers remains unclear and is generally thought to involve the IKK2-IκB-NF-κB signaling module that also allows for signal transduction of inflammatory stimuli (Figure 1A). As we previously recapitulated NF-κB/RelA activation in response to tumor necrosis factor receptor (TNFR) and Toll-like receptor (TLR)-4 stimulation in a mathematical model, we set out to examine the mechanism of NF-κB/RelA activation downstream of LTβR in a genetically tractable cell culture system to similarly reconstitute the underlying signaling mechanism in silico.

We stimulated mouse embryonic fibroblasts (MEF) with an agonistic antibody raised against LTβR that was shown to functionally complement for the genetic absence of LTαβ (Rennert et al., 1998). Using subsaturating concentrations of antibody that resulted in specific signaling events (Figures S1A–S1H), we detected nuclear NF-κB DNA binding activity (Figure 1B) that consisted of both RelA:p50 and RelB:p50 dimers (Figure 1C), as previously described (Derudder et al., 2003).

One of the critical steps in NF-κB/RelA activation in response to inflammatory stimuli is NEMO-dependent IKK phosphorylation and degradation of RelA bound IκBs. Immunoprecipitated IKK activity could be detected in response to very low concentrations (0.01 ng/ml) of TNF (Figure 1D, lanes 7–12), but, surprisingly, nonsaturating LTβR stimulation conditions did not elicit any NEMO-associated IKK activity (Figure 1D, lanes 18–22). Consistently, cytoplasmic immunoblotting revealed no decrease in IκB protein levels (Figure 1E, lanes 8–11) and no inducible IκBα phosphorylation (Figures S1I and S1J).

nfkb2 p100 Is an Inhibitor of NF-κB/RelA:p50 DNA Binding Activity

These observations led us to search for novel factors that regulate NF-κB/RelA dimers in response to noncanonical stimuli. To this end we utilized a cell line deficient in the three canonical IκB proteins, IκBα, -β, and -ε ($i\kappa b^{-/-}$). Initial biochemical characterization revealed that despite the loss of these known NF-κB inhibitors, only a small fraction of cellular RelA was constitutively nuclear, while the majority of RelA was still cytoplasmic (Figure 2A), consistent with observations in IκB knockdown cells (Tergaonkar et al., 2005). To investigate the molecular basis for cytoplasmic sequestration, we reconstituted a cell line also lacking the *rela* gene ($i\kappa b^{-/-}rela^{-/-}$) with a tandem

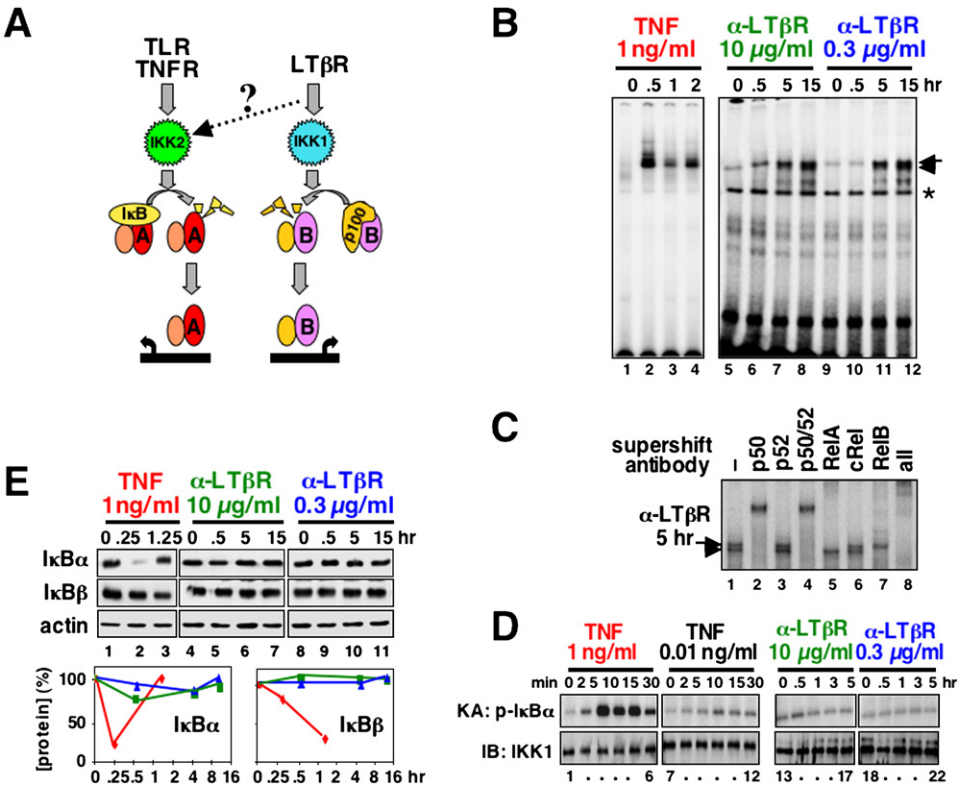

Figure 1. LTβR-Induced NF-κB/RelA Dimer Activation Does Not Correlate with Canonical IκB Kinase Activity or IκB Degradation

(A) A current model of NF-κB activation mechanisms resulting in RelA:p50 and RelB:p52 dimer activation via the canonical (IKK2) and the noncanonical (IKK1) pathway, respectively. The pathway that mediates NF-κB/RelA dimer activation in response to noncanonical stimuli has not been elucidated.

(B) Nuclear NF-κB DNA binding activities (arrows and arrowhead) induced by 1 ng/ml of TNF, 10 μg/ml or 0.3 μg/ml of LTβR agonistic antibody were resolved by EMSA with a κB-site containing probe. Asterisk denotes a nonspecific DNA-protein complex.

(C) The composition of NF-κB DNA binding activities induced upon LTβR receptor ligation was examined by supershift analysis using the indicated antibodies (lanes 2–8). At 5 hr poststimulation, RelA:p50 (arrow) and RelB:p50 (arrowhead) dimers were detected.

(D) IKK kinase activity was monitored by incubating GST-IκBα with anti-NEMO coimmunoprecipitates from extracts prepared from MEF stimulated with 1 ng/ml TNF, 0.01 ng/ml TNF, or with 10 μg/ml α-LTβR antibody, 0.3 μg/ml α-LTβR antibody. Reaction mixtures were resolved in SDS-PAGE and used for autoradiography (top) or immunoblotting (bottom) of coprecipitated IKK1 as a loading control.

(E) IκBα and -β immunoblot of extracts prepared from TNF- (red lines) or LTβR- (green and blue lines) stimulated cells. Signals were quantitated, normalized to the actin loading control, and graphed relative to signal levels in resting cells (bottom panel).

epitope-tagged form of RelA (TAP-RelA) by retrovirus-mediated transduction (Figure S2A).

Tandem affinity purification of epitope-tagged RelA in nondenaturing conditions followed by silver staining of purified proteins separated on SDS-PAGE as well as mass-spectrometric analysis revealed *nfkb1* p50 and *nfkb2* p100 as the two major interacting proteins (Figure 2B). Coimmunoprecipitation analysis confirmed that p100 was bound to RelA not only in IκBα/β/ε-deficient (Figures 2C and S2B, right panels) but also wild-type extracts (Figures 2C and S2B, left panels). Interestingly, a comparison of immunoprecipitates of RelA and RelB revealed that p100 was distributed evenly between RelB and RelA bound complexes in wild-type cells (Figure 2D).

We employed the detergent deoxycholate (DOC) to disrupt the interaction of endogenous IκB proteins and NF-κB (Baeuerle and Baltimore, 1988) and reveal the DNA binding activity (Figure 2E, compare lanes 1 and 2) of a RelA:p50 dimer (Figure S2C, top panel) in cytoplasmic extracts prepared from unstimulated wild-type MEF. As expected, prior immunodepletion of IκBα from the extract removed the majority of latent NF-κB DNA binding activity (lane 3). However, immunodepletion of p100 also resulted in a significant loss in DOC-inducible DNA binding activity, while immunodepletion of p105 did not (lanes 4 and 5). These results suggest that a fraction of cytoplasmic RelA:p50 dimer is not bound to canonical IκB proteins but to p100. Strikingly, treating $i\kappa b\alpha^{-/-}\beta^{-/-}\varepsilon^{-/-}$ cytoplasmic extracts with DOC liberated latent NF-κB DNA binding activity composed of RelA and p50 (Figures 2E, lanes 6 and 7, and S2C, bottom panel). Prior immunodepletion of p100 specifically eliminated almost all of the DOC-induced DNA binding activity (Figure 2E, lanes 8 and 9).

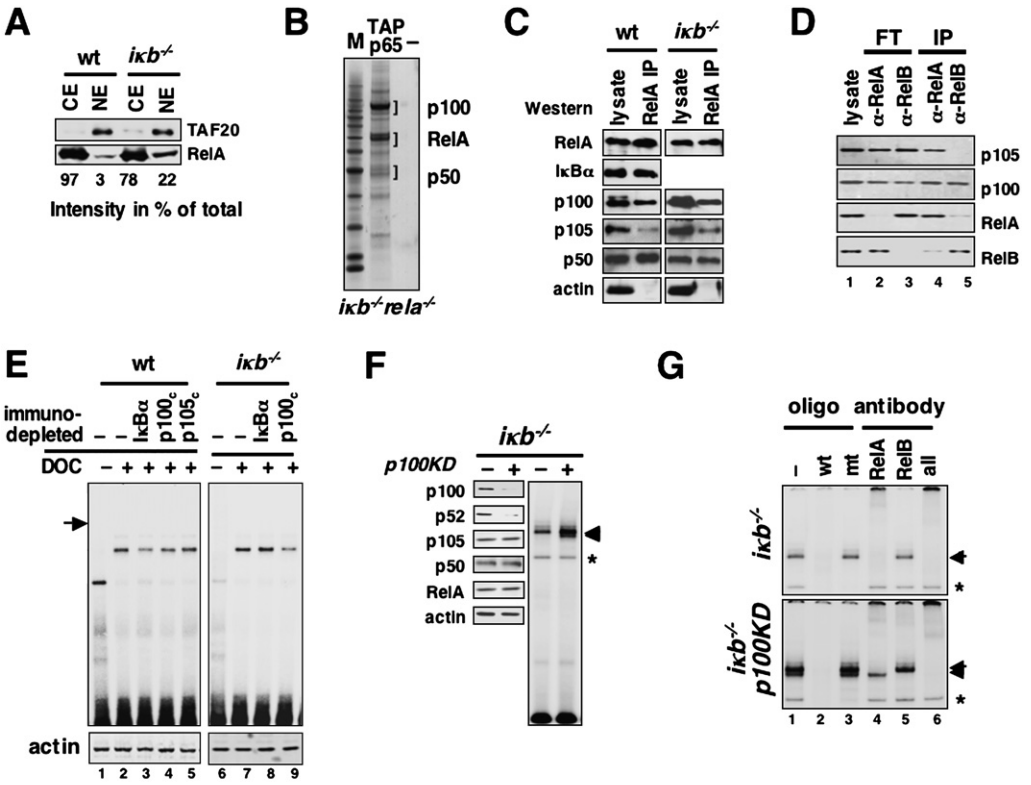

Figure 2. *nfkb2* p100 Is an Inhibitor of the RelA:p50 DNA Binding Activity

(A) Subcellular localization of RelA in wild-type and $i\kappa b\alpha^{-/-}\beta^{-/-}\varepsilon^{-/-}$ MEF ($i\kappa b^{-/-}$) was revealed by immunoblot of cytoplasmic (CE) and nuclear (NE) extracts. Signals were quantitated, and respective percentages of total are indicated below the blot. An immunoblot for the TFIID component TAF20 (a.k.a. TAF12) served as a fractionation control.

(B) Silver-stained SDS-PAGE of tandem affinity purified (TAP)- p65 protein expressed by a retroviral transgene in $i\kappa b\alpha^{-/-}\beta^{-/-}\varepsilon^{-/-}rela^{-/-}$ MEF. Mock purification from equivalent extracts prepared from the parental MEF confirms the specificity of the associated proteins. LC-MS/MS analysis revealed that indicated gel slices contained p100, p65, and p50 proteins respectively.

(C) Immunoblots of RelA coimmunoprecipitates prepared from wild-type (left panel) or IκBα/β/ε-deficient (right panel) MEF lysates revealed RelA association with IκBα, p50, p100, and p105 proteins. Lysate corresponding to half as many cells as the immunoprecipitate was probed as a control.

(D) The fractions of cellular p100 or p105 bound to RelA or RelB were examined by immunoblots of RelA or RelB immunoprecipitate (IP) and compared to 50% cell equivalent of crude lysate or flowthrough (FT).

(E) Latent cytoplasmic NF-κB DNA binding activity in wild-type (left panel) or IκBα/β/ε-deficient cells (right panel) were revealed by deoxycholate (DOC) treatment and EMSA (lanes 2 and 7). Prior immunodepletion with the indicated antibodies removed the target protein along with associated latent NF-κB activity. An immunoblot (bottom panel) against actin served as a loading control.

(F) IκBα/β/ε-deficient MEF were subjected to p100 knockdown by lentivirus-mediated delivery of a specific shRNA. Lysates prepared from resulting $i\kappa b^{-/-}$*p100KD* and parental $i\kappa b^{-/-}$ cells were compared by immunoblots for indicated proteins (left panel). Similarly, nuclear extracts were compared for NF-κB DNA binding activity by EMSA (right panel).

(G) Specificity and composition of the constitutive NF-κB DNA binding complexes present in the indicated nuclear extracts were determined by oligonucleotide competition (lanes 2 and 3) and supershift analysis with indicated antibodies (lanes 4–6).

These biochemical results prompted us to examine the RelA:p50-p100 interaction genetically. Lentiviral delivery of small hairpin RNA (shRNA) to "knockdown" *nfkb2* expression in IκBα/β/ε-deficient cells resulted in more than 90% reduction of p100/p52 protein levels (Figure 2F, left panel). Importantly, electrophoretic mobility shift assay (EMSA) with nuclear extracts prepared from these $i\kappa b^{-/-}$/*p100KD* cells showed an ~5-fold increase in the NF-κB activity over controls (Figures 2F, right panel, and S2E). Supershift analysis confirmed that the majority of this DNA binding activity consisted of RelA:p50 dimers (Figure 2G, data not shown). In sum, our results demonstrated that, analogous to the canonical IκB proteins, p100 is able to sequester RelA:p50 dimers in the cytoplasm and inhibit their DNA binding activity in a DOC-sensitive manner.

nfκb2 p100 Is a Stimulus-Responsive Regulator of NF-κB/RelA:p50 Activity

We sought to examine the stimulus-responsive behavior of p100 bound to RelA:p50 dimers. By immunoprecipitating RelA in nondenaturing conditions from cellular

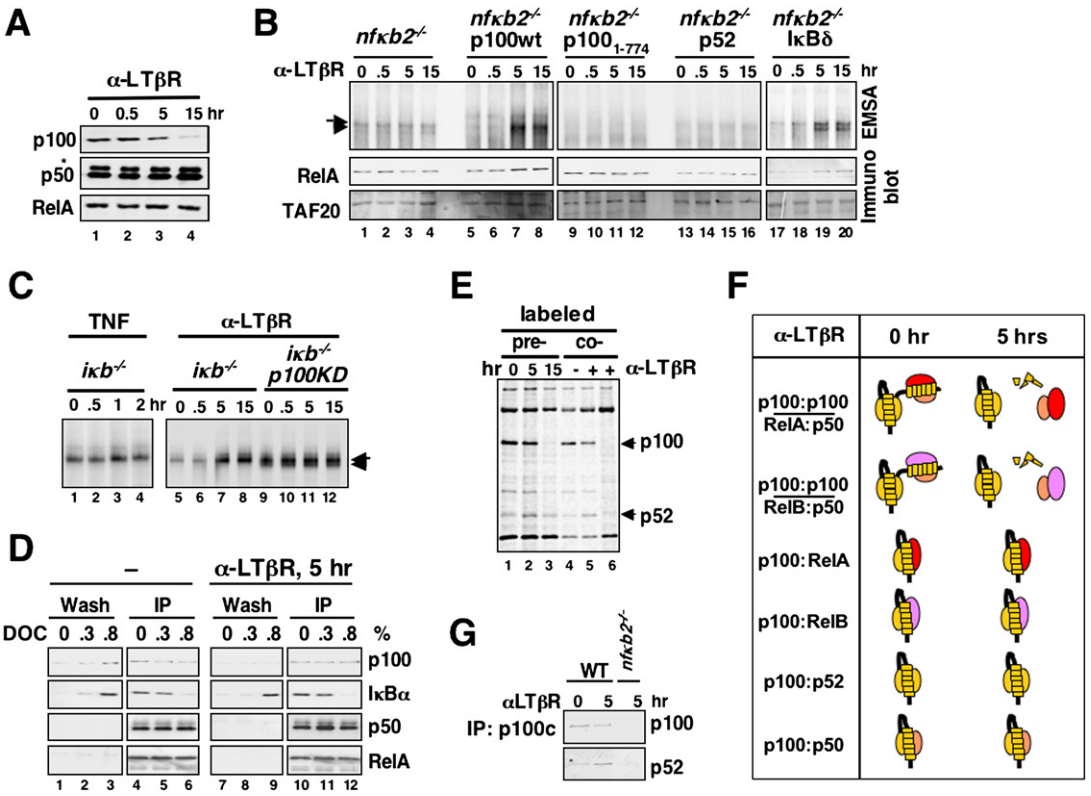

Figure 3. *nfkb2* p100 Is a Stimulus-Responsive Regulator of the NF-κB/RelA Dimer

(A) Association of p100 and RelA during LTβR signaling was monitored by p100 immunoblotting of RelA coimmunoprecipitates performed with extracts prepared from wild-type cells at the indicated times. Immunoblots for p65 and p50 served as a control for coIP efficiency. "*" indicates a nonspecific band.

(B) The requirement of specific domains of p100 in LTβR-induced NF-κB/RelA activation was examined. NF-κB/RelA activation was scored by EMSA (top panel) and by immunoblotting (bottom panels) nuclear extracts prepared from LTβR agonist treated *nfkb2*$^{-/-}$ MEF or *nfkb2*$^{-/-}$ MEF stably transduced with retrovirus expressing full-length p100, a mutant that lacks the C-terminal stimulus-responsive phosphorylation sites ("p100$_{1\text{-}774}$"), a C-terminal truncated protein that lacks the IκB-like domain ("p52"), or an N-terminal truncated protein that lacks RHD ("IκBδ").

(C) EMSA of NF-κB DNA binding induced in *iκbα*$^{-/-}$*β*$^{-/-}$*ε*$^{-/-}$ cells by TNFR (left panel) or LTβR (right panel, lanes 5–8) stimulation for the indicated times. The effect of p100 knockdown in *iκbα*$^{-/-}$*β*$^{-/-}$*ε*$^{-/-}$ cells on the inducibility of NF-κB DNA binding activity by LTβR stimulation was monitored similarly by EMSA (right panel, lanes 9–12).

(D) RelA interactions with p100 via the RHD or ARD were distinguished by deoxycholate (DOC) sensitivity. RelA-immunoprecipitates prepared from cytoplasmic extracts from resting (left panel) or LTβR agonist treated (right panel) cells were washed with increasing concentrations of DOC. Washes and immunoprecipitates were examined by immunoblotting with indicated antibodies.

(E) LTβR-induced proteolysis of pre-existing or de novo synthesized p100 protein was monitored using pulse-chase in vivo labeling. N-terminal p100 antibody was used in immunoprecipitates with extracts prepared from prelabeled cells at the indicated time points of an LTβR time course (lanes 1–3, "chase"), and from cells labeled during the later phase of mock or LTβR time course (lanes 4 and 5, "pulse"). Similarly treated extracts from *nfκb2*$^{-/-}$ cells (lane 6) served to identify nonspecific bands.

(F) Multiple *nfkb2* p100 NF-κB complexes are functionally distinct: we propose to distinguish between self-inhibited dimeric complexes of p100 and RelA or RelB, which are unresponsive to LTβR signaling, and stimulus-responsive ternary complexes in which the IκBδ domain of p100 inhibits functional RelA:p50 and RelB:p50 dimers in *trans*.

(G) Increased p52 association with p100 following LTβR signaling. Immunoblot for p52/p100 proteins of p100 C-terminal domain immunoprecipitates prepared from indicated cell extracts.

extracts, we found that LTβR stimulation led to a decrease in RelA-associated p100 protein (Figure 3A), suggesting that p100 may mediate NF-κB/RelA activation in response to LTβR stimulation. Using a genetic approach, we found that LTβR stimulation of *nfκb2*$^{-/-}$ cells were not only deficient in RelB activation as reported (Derudder et al., 2003; Lo et al., 2006) but that NF-κB/RelA activation was also abrogated (Figure 3B, top panel, lanes 1–4). Reconstitution of *nfkb2*$^{-/-}$ MEF with retrovirally expressed p100 restored LTβR-inducible RelA and RelB DNA binding activities and RelA translocation (Figure 3B, lanes 5–8) concomitant with p100 degradation (Figure S3A). Interestingly, reconstitution of *nfkb2*$^{-/-}$ cells with the processed p52 protein did not restore signal inducible RelA nuclear activity indicating the requirement for the IκB-like ankyrin repeat domain of p100 (Figure 3B, lanes 13–16). Further,

a mutant form of p100 lacking the C-terminal signal responsive phosphorylation sites also did not functionally complement the knockout cells (Figure 3B, lanes 9–12). Strikingly, expression in *nfkb2*$^{-/-}$ MEFs of the C-terminal portion of p100 containing the ankyrin repeat domain (IκBδ) was sufficient for at least partial RelA:p50 activation upon LTβR ligation (Figure 3B, lanes 17–20).

To examine whether *nfkb2* p100 is sufficient for NF-κB/RelA activation or the canonical IκB proteins are also required, *iκbα*$^{-/-}$*β*$^{-/-}$*ε*$^{-/-}$ cells were examined in stimulation time courses. While the elevated nuclear RelA:p50 activity in these cells was not further inducible by TNF (Figure 3C, lanes 1–4), LTβR stimulation resulted in strong induction of both nuclear RelA and RelB proteins and DNA binding activities with kinetics that were similar to those observed in wild-type cells (Figures 3C, lanes 5–8, and S3B–S3D). Activation of NF-κB binding activities in *iκbα*$^{-/-}$*β*$^{-/-}$*ε*$^{-/-}$ cells was accompanied by loss in cellular and RelA-associated p100 protein (Figures S3E and S3F). Furthermore, we found that LTβR signaling to NF-κB was abrogated in *iκb*$^{-/-}$*/p100KD* (Figure 3C, lanes 9–12). Collectively, these analyses provide biochemical and genetic evidence for the requirement and sufficiency of p100 as a stimulus-specific regulator of RelA dimers in response to noncanonical signaling triggered by LTβR stimulation.

Multiple *nfkb2* p100 NF-κB Complexes Are Functionally Distinct

Unlike canonical IκBs, *nfkb2* p100 is not only capable of interacting with RelA:p50 dimers in *trans* via its IκBδ domain as revealed by DOC-treated extracts analyzed by EMSA (Figure 2E), it can also dimerize with other NF-κB proteins via its Rel homology domain (RHD). Thus p100 is capable of forming complexes with NF-κB of distinct molecular architectures. To characterize the architecture of the complex(es) relevant for noncanonical signaling, we mapped DOC-sensitive and -insensitive interactions between RelA and p100 during LTβR signaling (Figure 3D). In resting cells (lanes 1–6), we found that most but not all p100 could be DOC-stripped off immunoprecipitated RelA proteins (top panel). In contrast, in LTβR-stimulated cells (lanes 7–12), no DOC-sensitive p100 could be detected in the RelA immunoprecipitate, while the DOC-resistant fraction remained. Efficient stripping of IκBα and retention of p50 in the RelA immunoprecipitate confirmed the specificity of the DOC strip conditions. Similar analyses of canonical IκBα/β/ε-deficient cells (Figure S3G) also revealed that DOC-sensitive p100-RelA complexes are responsive to LTβR signaling.

Given that the IκBδ domain is genetically sufficient for LTβR induction of RelA:p50 dimer (Figure 3B), we examined biochemically the fate of the p100 RHD during noncanonical signaling. To this end, cellular proteins were pulse labeled with ^{35}S-methionine before adding the receptor agonist. Immunoprecipitates obtained with an N-terminal p100 RHD antibody revealed ^{35}S-labeled p100 prior to stimulation (Figure 3E, lane 1). Interestingly, LTβR stimulation led to the conversion of this pre-existing p100 to a polypeptide (lane 2) of the same molecular weight as the p52 generated de novo during LTβR signaling (lane 5). However, while de novo generated p52 protein participates in the LTβR-induced NF-κB DNA binding activities (Figure S1C), the p52 protein generated from pre-existing p100 complexes does not (Figures 1C and S3C).

To summarize our data we propose the following molecular architectures of p100 complexes (Figure 3F). LTβR-unresponsive complexes of p100 include the previously identified "self-inhibited" p100:RelA, p100:RelB, p100:p50, and p100:p52 dimers in which the IκBδ domain of p100 folds back onto the dimer to inhibit its DNA binding activity in *cis* (Beinke and Ley, 2004; Mercurio et al., 1993; Naumann et al., 1993). In addition, two p100 proteins may form a necessarily asymmetric dimer, analogous to two p105 proteins (Moorthy and Ghosh, 2003; Moorthy et al., 2006). In that complex, one of the IκBδ domains would inhibit the DNA binding activity of the p52 homodimer, while the other IκBδ domain would be available to inhibit a RelA:p50 or a RelB:p50 dimer. Our results suggest that this second IκBδ domain is sensitive to LTβR signaling to undergo proteolysis leaving the C-terminal RHD intact. The remaining p100:p52 complex would be self-inhibited and therefore incapable of participating in NF-κB DNA binding activity. However, during the later phase of LTβR signaling de novo synthesized p52 dimerizes with Rel proteins via the RHD and contributes to nuclear DNA binding activity as previously observed (Dejardin et al., 2002; Muller and Siebenlist, 2003).

Based on these proposed functional roles of distinct p100 complexes, we would predict that the amount of p52 protein associated with p100 increases during the early phase of LTβR signaling. Indeed, immunoprecipitation of p100 with an antibody specific for the C-terminal IκBδ domain yields more coprecipitating p52 protein at the 5 hr time point of LTβR signaling than in resting cells (Figure 3G).

Noncanonical Signaling to NF-κB/RelA Activation

Activation of RelB dimers in the noncanonical signaling pathway was shown to depend on signal-responsive NIK-dependent activation of IKK1 (Beinke and Ley, 2004; Senftleben et al., 2001; Xiao et al., 2001). We asked if IKK1 is also required for activation of RelA dimers in response to LTβR signaling. Consistent with previous analyses (Derudder et al., 2003), *ikk1*$^{-/-}$ MEF did not show processing of p100 upon LTβR activation, and this could be restored upon retroviral reconstitution (Figure S4A). Whereas TNF activation of NF-κB was unaffected (Figure 4A, bottom panel), we did not detect inducible NF-κB activity containing either RelA or RelB in IKK1-deficient cells in response to LTβR signaling (top panel). Similarly, MEF derived from NIK knockout mice or from the alymphoplasia mutant mouse strain (NIK-*aly*), which contains an inactivating mutation in NIK (Shinkura et al., 1999), showed not only a complete abrogation of RelB but also RelA dimer activation upon LTβR stimulation (Figures 4B and S4B). Furthermore, shRNA mediated

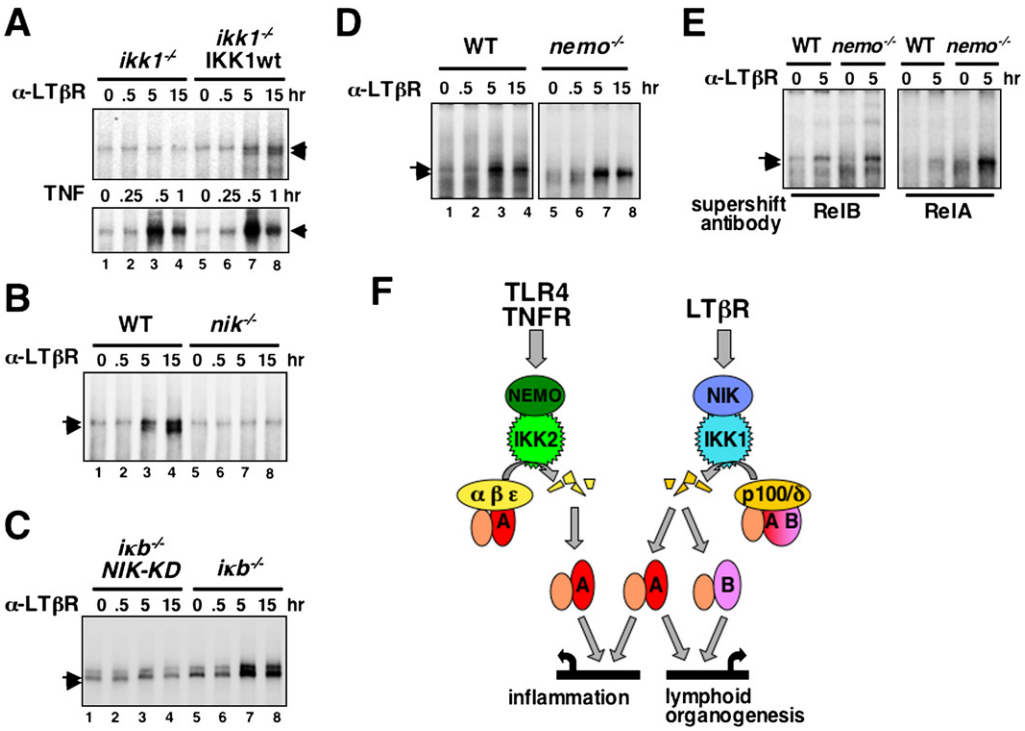

Figure 4. Signal Transducers of the Noncanonical Pathway Are Required for LTβR-Induced RelA Dimer Activation

(A) LTβR- and TNF-induced NF-κB DNA binding activities were monitored by EMSA in *ikk1*$^{-/-}$ MEF reconstituted with an IKK1-expressing retrovirus (lanes 5–8) and parental IKK1-deficient MEF (lanes 1–4). Arrows indicate specific NF-κB-DNA complexes.

(B) NF-κB DNA binding activities induced by LTβR stimulation in *nik*$^{-/-}$ (lanes 5–8) and control wild-type (lanes 1–4) MEF was monitored by EMSA.

(C) NF-κB DNA binding activities induced by LTβR stimulation in *iκbα*$^{-/-}$*β*$^{-/-}$*ε*$^{-/-}$ MEF (lanes 5–8) or such IκBα/β/ε-deficient cells in which NIK expression was knocked down by lentiviral shRNA expression were monitored by EMSA.

(D) The requirement for NEMO in LTβR activation of NF-κB was examined by EMSA of LTβR stimulated *nemo*$^{-/-}$ MEF.

(E) Induction of RelA- and RelB-containing dimers was assessed by immuno-ablation of the complexes with the indicated supershift antibody.

(F) A schematic model of two pathways leading to the activation of NF-κB/RelA:p50 activity. The canonical IκB proteins IκBα, -β, and -ε mediate RelA:p50 activation in response TNFR stimulation downstream of NEMO and IKK2. We propose that the noncanonical IκB protein p100/IκBδ inhibits the DNA binding activity of both RelA:p50 and RelB:p50 dimers and mediates their activation in response to LTβR stimulation downstream of NIK and IKK1.

knockdown of NIK by lentiviral vectors in *iκbα*$^{-/-}$*β*$^{-/-}$*ε*$^{-/-}$ cells (Figure S4C) dramatically reduced RelA activation in response to LTβR engagement (Figure 4C). These results support the notion that noncanonical signaling via NIK and IKK1 regulates the degradation of *nfkb2* p100 to affect NF-κB/RelA activation.

A hallmark of canonical NF-κB signaling is the requirement for NEMO in inflammatory stimulus-responsive degradation of canonical IκB proteins. However, p100 processing to p52 upon LTβR activation was found to be intact in *nemo*$^{-/-}$ MEF (Dejardin et al., 2002; Muller and Siebenlist, 2003). Our EMSA analysis confirmed that *nemo*$^{-/-}$ MEF fail to induce NF-κB activity upon TNF treatment (Figure S4D), but we found strong induction of NF-κB DNA binding activity in response to LTβR treatment (Figure 4D). DNA binding analysis performed in the presence of RelB or RelA supershift antibody revealed that both RelA:p50 and RelB:p50 activities remained inducible in the absence of NEMO (Figures 4E and S4E).

Our results suggest a new model for the activation mechanism of NF-κB/RelA dimers in response to LTβR signaling. While LTβR signaling was proposed to bifurcate into the NEMO-IKK2-IκB-RelA and the IKK1-p100-RelB axes, our new understanding of the pathway suggests that p100 is associated with pre-existing RelA:p50 and RelB:p50 dimers (Figure 4F). NIK and IKK1 regulate the signal-responsive proteolysis of p100/IκBδ, which results in nuclear RelA:p50 and RelB:p50 DNA binding activity in a NEMO-independent manner. Continued synthesis and processing of p100 to p52 may enhance the level of p52 containing NF-κB DNA binding complexes at later time points (Dejardin et al., 2002; Muller and Siebenlist, 2003).

By investigating the mechanisms responsible for RelA dimer activation upon LTβR stimulation, we identified p100 as a stimulus-selective signal transducer that satisfies two criteria of bona fide IκB proteins: the ability to (1) sequester latent NF-κB dimers and (2) release the bound dimers via stimulus-induced proteolysis. While

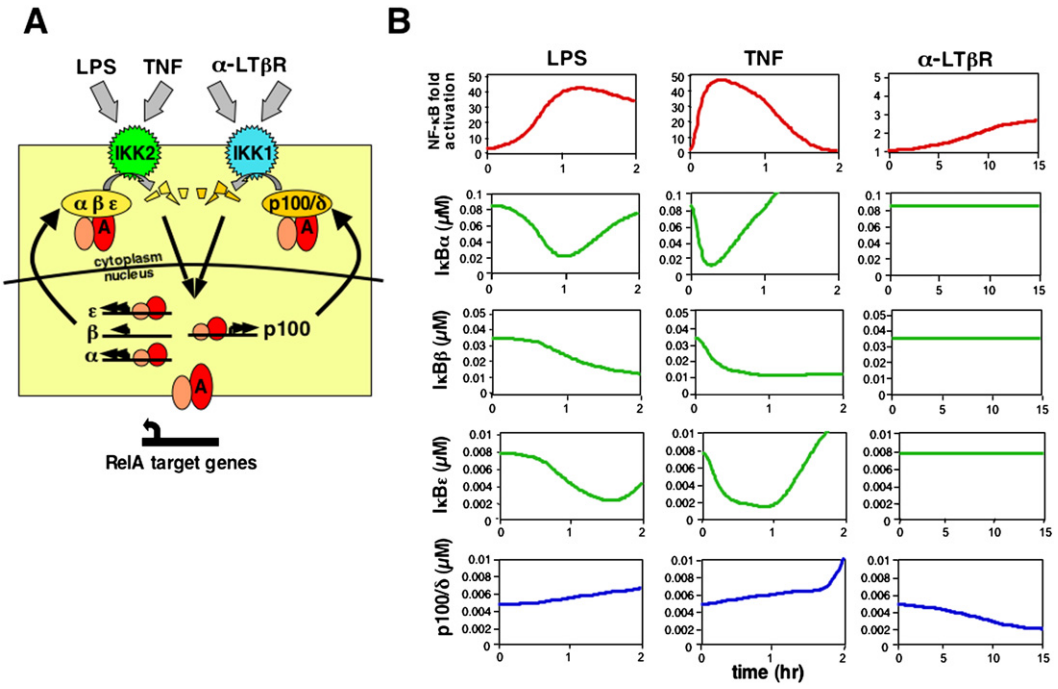

Figure 5. A Single NF-κB Signaling Module Mediates NF-κB RelA Activation in Response to Inflammatory and Developmental Signals

(A) A schematic presentation of the NF-κB signaling module that includes three canonical IκB proteins IκBα, -β, and -ε that mediate signals from IKK2-inducing stimuli and the noncanonical IκB protein p100/δ that mediates signals from IKK1 inducing stimuli. IκBα, IκBε, and p100 are encoded by NF-κB response genes and are able to provide negative feedback. Biochemical reactions encompassed in the yellow box are represented as ordinary differential equations in a mathematical model (version 3.0, see Supplemental Data).

(B) Computational simulations of the dynamic regulation of nuclear RelA:p50 activity (top panels, red), total cellular protein levels of canonical IκB proteins (middle panels, green) and noncanonical IκB protein p100 (bottom panels, blue). Simulations are shown for the IKK2-inducing inflammatory stimuli TNF (left column) and LPS (center column) and the IKK1-inducing developmental signals mediated by LTβR (right column).

p100/IκBδ is a bona fide IκB protein, it differs from the canonical IκB proteins IκBα, -β, and -ε in its ability to sequester not only RelA- but also RelB-containing dimers and in its responsiveness to noncanonical (e.g., LTβR, BAFFR) rather than canonical (e.g., TNFR and TLR) signaling pathways. Thus, we propose to distinguish between the canonical IκB proteins IκBα, -β, and -ε and the noncanonical IκB protein p100/IκBδ.

Mathematical Reconstruction of Noncanonical Signaling to NF-κB/RelA

Our new understanding expands the NF-κB signaling module to include *nfkb2* p100/IκBδ as a fourth IκB that controls NF-κB/RelA activity in response to noncanonical, IKK1-inducing stimuli (Figure 5A). Studies of the dynamic behavior of the canonical IκB proteins have resulted in important insights about NF-κB signaling and stimulus-specific gene expression (Hoffmann et al., 2002; Kearns et al., 2006; Werner et al., 2005) in response to inflammatory stimuli. To investigate how the dynamics of p100 regulation affect RelA activation in response to noncanonical signaling, we described the synthesis, degradation, and molecular interactions of p100 with ordinary differential equations (see Supplemental Data) and included them in a mathematical model that already recapitulated TNF and LPS signaling. The resulting model (version 3.0) includes 98 biochemical reactions, which were parameterized based on published measurements, our own measurements, and fitting procedures. For p100 in particular, we measured mRNA and protein levels and their half-lives in resting cells and in response to NF-κB inducing stimuli (V. Shih, J.K., S.B., A.H., data not shown). Transcriptional and translational synthesis and degradation rate constants were fitted to these measurements.

The resulting in silico model of the NF-κB signaling module predicts NF-κB/RelA:p50 activity in response to either canonical or noncanonical stimuli, as well as the abundance of the 31 model components including the IκB proteins, free or complexed to IKK or NF-κB, in either the cytoplasm or the nucleus (Figure S5 and Supplemental Data). Simulations of LPS and TNF pulse stimulations show the dynamics of NF-κB activities and cellular canonical IκB levels (Figure 5B, left and center columns) that match previous measurements (Werner et al., 2005). Unlike the canonical IκB proteins, NF-κB p100 is not degraded in response to IKK2-mediated inflammatory signals (Figure 5B, bottom row). In contrast, IKK1-mediated LTβR signaling results in p100 degradation while the canonical IκB

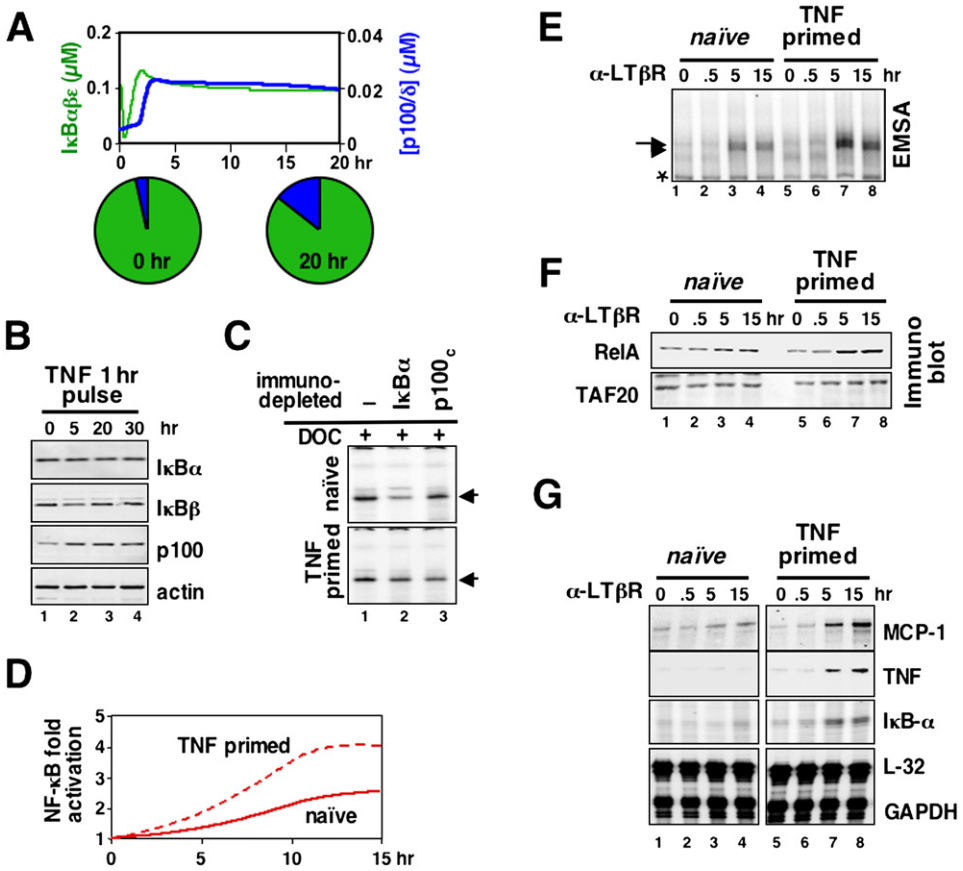

Figure 6. *nfkb2* p100 Mediates Crosstalk between Inflammatory and Developmental Signals

(A) Cellular levels of canonical (IκBα, -β, and -ε; green) and noncanonical (*nfκb2* p100, blue) IκB proteins over a 20 hr time course as predicted by computational simulations of cells stimulated with a 1 hr pulse of TNF. The pie charts indicate the proportion of NF-κB/RelA bound to canonical IκBs (green) versus the noncanonical IκB (blue) in resting (left) versus TNF-primed (right) cells.

(B) Immunoblots for canonical IκBα and -β and noncanonical p100/δ protein of whole-cell lysates prepared at the indicated times from wild-type MEF that were transiently stimulated with 1 ng/ml of TNF for 1 hr.

(C) EMSA for NF-κB activity liberated by deoxycholate (DOC) treatment of cytoplasmic extracts prepared from naïve (top panel) or TNF-primed (bottom panel) MEF. Prior to DOC treatment extracts were either mock, IκBα, or p100/δ-immunodepleted. RelA:p50 DNA binding complexes are indicated by the arrow.

(D) Computational simulations of RelA:p50 activity induced by LTβR stimulation of naïve or TNF-primed cells.

(E) Experimental analysis of LTβR signaling in naïve or TNF-primed cells. Cells were harvested at indicated time points after LTβR stimulation, and nuclear extracts were tested for RelA:p50 DNA binding activity by EMSA.

(F) Immunoblotting of nuclear RelA protein (top) during LTβR time course in naïve and TNF-primed cells. An immunoblot for TAF20 (bottom) served as a loading control.

(G) RNase protection assay to monitor the expression of NF-κB response genes induced by LTβR stimulation in naïve (left) and TNF-primed cells (right).

proteins are unaffected (Figure 5B, right column). Most importantly, the model recapitulates our measurements of slow induction of nuclear NF-κB/RelA activity that reaches a maximum at 5–15 hr (Figure S1B).

Crosstalk between Canonical and Noncanonical Signaling

Given that *nfkb2* p100, like IκBα and IκBε (Kearns et al., 2006), is an NF-κB target gene, we used computational simulations to explore feedback regulation and the dynamics of p100 protein levels in a variety of stimulation regimes. Remarkably, we found that a 1 hr pulse of TNF stimulation resulted in an elevation of p100 protein of about 4-fold that persisted for more than 20 hr (Figure 6A, top graph). In contrast, graphing the sum of all canonical IκB proteins revealed the expected transient trough and rapid recovery. In sum, the model predicted that the fraction of p100-associated RelA protein at the 20 hr time point to be increased 3- to 4-fold (Figure 6A, bottom graph).

We examined these predictions experimentally. Immunoblotting of total cellular IκB protein confirmed that p100 protein is induced by 1 hr TNF pulse stimulation, while the levels of canonical IκB proteins were not discernibly different (Figure 6B). By quantitating IκB-NF-κB

interactions with the DOC-EMSA assay coupled to immunodepletions, we found that TNF-primed cells contained much more p100-NF-κB complexes than unstimulated/naïve cells (Figure 6C). These data confirm the model's prediction that priming of cells can shift the balance of latent NF-κB associated with canonical or noncanonical IκB proteins.

This shift in the IκB homeostasis may have functional consequences. Computational simulations of LTβR signaling in naïve and TNF-primed cells led to the prediction that primed cells may respond with a stronger NF-κB activation profile (Figure 6D). This prediction is remarkable because in the simulated stimulation regimen canonical signaling has long ceased when cells are exposed to agonist antibody, and it thus not only constitutes an example of signaling crosstalk but also of cellular memory. We examined the prediction experimentally. Our EMSA and nuclear Immunoblot results confirmed that 2- to 3-fold more NF-κB/RelA activity is induced by LTβR stimulation in cells that were previously primed with TNF (Figures 6E and 6F).

We investigated the gene-expression effects of this elevated NF-κB response using quantitative multiplex RNase protection assays. While naïve MEF did not show appreciable inflammatory gene activation in response to the LTβR stimulation, primed MEF exhibited induction not only of the IκBα gene, a hallmark of the canonical NF-κB response, but also the gene encoding the inflammatory cytokine TNF (Figure 6G). These results constitute qualitative changes in stimulus-responsive gene expression programs. Our computational simulations of the NF-κB signaling module revealed that the homeostatic control of IκB signal transducers integrates the history of cellular exposure to NF-κB inducing stimuli. Our experimental results confirmed the validity of the proposed model and resultant crossregulatory mechanism between canonical and noncanonical NF-κB inducing signals and revealed that its functional consequences for stimulus-specific gene expression programs may be profound.

Genetic Perturbations of IκB Homeostasis

The homeostasis of canonical and noncanonical IκB proteins may not only be subject to physiological stimuli, but altered homeostasis due to genetic aberrations may be the underlying cause for pathological misregulation of stimulus-specific gene expression. To examine this possibility and further test the validity of our mathematical model, we used computational simulations to predict the relative amounts of p100 protein in a panel of cells deficient for one or more canonical IκB proteins. For example, cells deficient in IκBα were predicted to have 2-fold more p100 protein than wild-type cells (Figure 7A). Similarly, doubly-deficient and triply-deficient cells are predicted to show further increases in p100 except for IκBβ/ε-deficient cells, in which p100 levels were predicted to be near wild-type. Immunoblotting for p100 with extracts derived from these different cell lines confirmed this prediction (Figure 7B); in particular, we observed no significant increases in p100 levels in IκBβ/ε-deficient cells while immunoblot signals were elevated in other knockouts as predicted.

Altered balances in the canonical and noncanonical IκB proteins may be expected to lead to alterations in the LTβR responsiveness. Computational simulations indeed predicted that NF-κB/RelA responses to agonist receptor would be highest in IκB triple-knockout and IκBα/ε-deficient cells, intermediate in IκBα- and IκBα/β-deficient cells, and relatively low in wild-type and IκBβ/ε-deficient cells (Figure 7C). The results from experimental analysis using EMSA of nuclear extracts prepared from LTβR-stimulated cells of each respective genotype were in overall agreement with these simulations (Figure 7D). These results suggested that genetic alterations in cells that constitutively alter the homeostasis of canonical and noncanonical IκB proteins affect the stimulus-specific activation of NF-κB. To determine whether these changes are functionally relevant, we examined the expression of known NF-κB response genes. Indeed, we found that cells that showed hyperresponsive NF-κB/RelA activation to LTβR stimulation also exhibited inflammatory gene expression, such as those encoding iNOS, MIP-2, IP-10, KC, or GM-CSF, not normally seen in wild-type MEF (Figure 7E). These effects were not merely quantitative but qualitative. Of the six target genes we examined we found a range of responsiveness that suggests that NF-κB target genes may have different threshold requirements for NF-κB activity that allow for differential regulation.

Canonical and Noncanonical IκBs in Physiology and Pathology

Our results offer a significant revision of our understanding of NF-κB signaling. By examining the mechanism for NF-κB/RelA activation in response to LTβR signaling, we identified *nfκb2* p100 as the pertinent signal transducer that functions as a bona fide IκB molecule. We propose that as a feedback regulator of the RelA:p50 transcriptional activator the noncanonical IκB protein p100 represents an integrator of canonical and noncanonical signals. The resulting signaling crosstalk between inflammatory and developmental stimuli suggests that the canonical and noncanonical NF-κB signaling pathways (previously described as separate) ought to be considered as being contained within a single signaling module. The "four IκB" computational model that integrates IKK1- and IKK2-mediated signals represents a first attempt in that direction. Future efforts will have to quantitatively address the regulation of RelB complexes and the generation of p52-containing dimers.

Within their physiological environment, cells receive multiple signals, and thus the potential for signaling crosstalk is significant. Relevant to lymphnode development, constitutive TNF signaling has been observed in the spleen (Schneider et al., 2004). Indeed, the in vitro generation of fibroblast reticular cells from lymphnode tissue requires both TNFR and LTβR stimulation (Katakai et al., 2004), while abrogation of TNF signaling in vivo results in

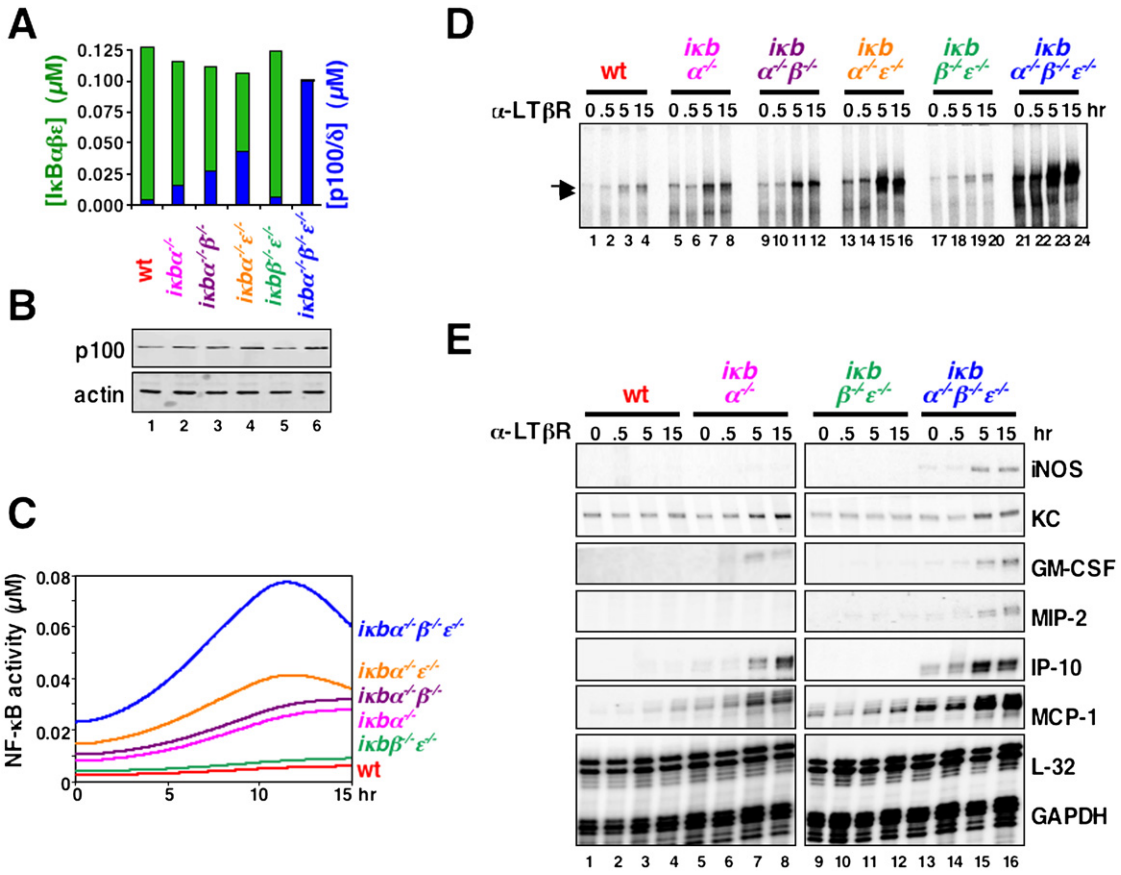

Figure 7. Misregulation of Canonical and Noncanonical IκB Homeostasis Alters Stimulus-Specific Gene Expression

(A) Cellular protein levels of canonical (IκBα, -β, and -ε; green) and noncanonical (*nfκb2* p100, blue) IκB proteins in unstimulated cells of the indicated genotype as predicted by computational simulations.

(B) Quantitative immunoblots for p100 of lysates prepared from equivalent number of wild-type or mutant MEF deficient of the indicated genotype.

(C) Computational simulations of nuclear RelA:p50 activity induced by LTβR stimulation in cells of the indicated genotype.

(D) EMSA for NF-κB binding activity of nuclear extracts prepared from LTβR-stimulated cells of the indicated genotype. Wild-type or mutant MEF that lack one or more IκB isoforms were stimulated with LTβR for the indicated times.

(E) RNase protection assay to monitor the expression of the indicated NF-κB response genes induced by LTβR stimulation in cells of the indicated genotype.

the loss of lymphnode architecture (Rennert et al., 1998). Fundamentally, all cells are continuously subject to low or varying amounts of TLR/TNFR signaling, albeit these conditions are not usually reproduced in sterile in vitro cell-culture conditions. Recent in vivo studies suggest that naïve T cell responses are regulated by integrating signals from memory and regulatory T cells via p100 (Ishimaru et al., 2006). Remarkably, *nfκb2* p100-mediated crosstalk does not have to occur coincidently but integrates the history of cellular exposure to inflammatory signals.

Conversely, our studies demonstrate that alterations in the homeostasis of IκB proteins can result in inappropriate expression of inflammatory genes in response to developmental/noncanonical LTβR signaling. We utilized defined genetic mutants with altered IκB protein expression patterns. These cells may serve as model systems for disease-associated misregulation. In this context, it is of interest to note that a significant fraction of malignant Reed Sternberg cells in Hodgkin's lymphoma has defective IκBα genes (Krappmann et al., 1999). Our findings suggest that pathogenic misregulation of gene expression in Reed Sternberg cells may in part be the result of noncanonical developmental signals impinging on an NF-κB signaling module with an altered homeostasis of IκB proteins. We suggest that the integration of multiple signals via signaling crosstalk represents an opportunity for therapeutic intervention, which may be more effectively exploited through the combined tools of computational modeling and biochemical analysis.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents

Primary and 3T3 immortalized MEF were generated from E12.5–14.5 embryos and maintained as previously described (Hoffmann et al., 2003). Generation of *iκbα*$^{-/-}$*β*$^{-/-}$*ε*$^{-/-}$ and *iκbα*$^{-/-}$*β*$^{-/-}$*ε*$^{-/-}$*rela*$^{-/-}$ cell

lines will be described elsewhere. Agonistic LTβR monoclonal antibody raised in hamster (AF.H6) was obtained from Jeff Browning (Biogen, Inc). Two other previously used Rat anti-LTβR antibodies, 3C8 and 4H8 (Dejardin et al., 2002), were also used (Figure S1F). Recombinant murine TNF was from Roche. RelA/p65 (sc-372), RelB (sc-226), cRel (sc-71), IκBα (sc-371), IκBβ (sc-946), and NIK (H-248) antibodies were from Santa Cruz Biotechnology. Phospho-IκBα (Ser-32 and Ser-36) monoclonal antibody was from Cell Signaling Technology. N-terminal p52 (1495), p50 (1157 and 1263) as well as C-terminal anti-sera for p100 (1310) and p105 (1140) were a generous gift from Nancy Rice. Antiserum against TAF20 was used to test nuclear fractionation. Sc-372G antibody was used for RelA immunoprecipitation and sc-226 crosslinked to protein-A-sepharose beads was used for RelB immunoprecipitate. Supershift antibodies have been described previously (Hoffmann et al., 2003).

Immunoblot, Immunoprecipitation, and Kinase Assay

Whole-cell extracts were prepared in RIPA buffer and normalized before immunoblot analysis. For immunoprecipitation-western analysis, whole-cell lysate from 10^6 cells was prepared in buffer containing 20% glycerol, 0.2 mM EDTA, 0.5% NP-40, and 150 mM NaCl. Band intensities were quantitated by phosphor-imager and normalized against the respective actin band. To evaluate the DOC sensitivity of RelA-p100 interactions, RelA immunoprecipitates obtained from cytosolic extract were washed with buffer containing 10 mM HEPES-KOH, pH 7.9, 150 mM KCl, 1 mM EDTA, and 0.1% NP-40 containing 0%, 0.3%, or 0.8% deoxycholate. Washes and SDS sample buffer-eluted immunopellets were immunoblotted. IKK kinase assays were performed as described (Werner et al., 2005).

Pulse-Chase Metabolic Labeling

In vivo pulse labeling of MEF was done with 100 μCi/ml trans ^{35}S-Met label (MP Biomedicals, Inc.) using the indicated time courses. p52/p100 were immunoprecipated (N-terminal antiserum 1495) from RIPA lysate. Proteins were resolved in 8% SDS-PAGE and visualized by autoradiography.

Retrovirus Mediated Gene Transduction

IKK1 and p100, $p100_{1\text{-}774}$ or p52 expressing retroviral constructs were gifts from M. Karin and A. Rabson. pBabe.IκBδ.puro was constructed by PCR amplifying C-terminal region of p100 encoding amino acids (483–934) using pBabe.p100.puro as template. Retroviral constructs were cotransfected with pCL.Eco into 293T cells, and 42 hr posttransfection filtered supernatant was used to infect MEF. Transduced cells were selected with puromycin hydrochloride (Sigma).

Lentiviral Delivery of shRNA

Lentiviral constructs expressing shRNAs against *nfkb2* and *nik* genes and control oligos were packaged into viruses as described previously (Tergaonkar et al., 2005). Sequences for specific oligo used in knockdown studies are available upon request. Four different control shRNA were used (Figure S2E) to ensure functional specificity.

TAP-p65 Complex Purification and Mass Spectrometry

Murine *rela* gene was cloned into pRAV-Flag-TAP bicistronic retroviral vector that expresses EGFP from an internal ribosome entry site (IRES) promoter (Knuesel et al., 2003). Tagged-*rela* gene was transduced into $ikb\alpha^{-/-}\beta^{-/-}\varepsilon^{-/-}rela^{-/-}$ cell line, and stable expressors were obtained by GFP-mediated fluorescence cell sorting. IgG binding domain-tagged p65 and interacting proteins were immunoprecipitated from the cell extract (from ~10^8 cells) using IgG-Sepharose 6 Fast Flow resin (Amersham Biosciences). Flag-RelA was eluted from the immunopellet by TEV-protease cleavage (Invitrogen) and reprecipitated with anti-Flag M2 affinity gel resin (Sigma). RelA complexes were eluted with 1 mg/ml Flag peptide (Sigma) and were analyzed by SDS-PAGE. Gel slices were cut, and in-gel trypsin digested and analyzed by LC-MS/MS at the Scripps Research Institute Center for Mass Spectrometry and searched against MASCOT/BLAST.

DNA Binding Assays

EMSAs were done as described with a κB site containing probe that binds to both RelA and RelB dimer with similar affinities (Lo et al., 2006). Super-shift assays were done with antibodies as described before (Hoffmann et al., 2003). For DOC sensitivity, 2 μg of cytosolic extract was treated with final 0.8% DOC for 30 min and subsequently subjected to EMSA analysis. Where specified, cytosolic extracts were immunodepleted for indicated protein prior to DOC treatment.

RNase Protection Assays

Total RNA was extracted from stimulated fibroblast using Trizol reagent. RPA was performed with 5 μg RNA using Riboquant probe set (BD-Bioscience) according to manufacturers' protocols.

Computational Modeling

The previously described mathematical model (Werner et al., 2005) was extended to include *nfκb2* p100 and noncanonical signaling via IKK1 as shown in Figure S5 and described in detail in the Supplemental Data. Simulations were done in Matlab version 2006a (Mathworks) using the built-in *ode15s* solver at default settings.

Supplemental Data

Supplemental Data include five figures, four tables, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at http://www.cell.com/cgi/content/full/128/2/369/DC1/.

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REFERENCES

Alcamo, E., Hacohen, N., Schulte, L.C., Rennert, P.D., Hynes, R.O., and Baltimore, D. (2002). Requirement for the NF-kappaB family member RelA in the development of secondary lymphoid organs. J. Exp. Med. *195*, 233–244.

Baeuerle, P.A., and Baltimore, D. (1988). I kappa B: a specific inhibitor of the NF-kappa B transcription factor. Science *242*, 540–546.

Beinke, S., and Ley, S.C. (2004). Functions of NF-kappaB1 and NF-kappaB2 in immune cell biology. Biochem. J. *382*, 393–409.

Bonizzi, G., Bebien, M., Otero, D.C., Johnson-Vroom, K.E., Cao, Y., Vu, D., Jegga, A.G., Aronow, B.J., Ghosh, G., Rickert, R.C., and Karin, M. (2004). Activation of IKKalpha target genes depends on recognition of specific kappaB binding sites by RelB:p52 dimers. EMBO J. *23*, 4202–4210.

Browning, J.L., Allaire, N., Ngam-Ek, A., Notidis, E., Hunt, J., Perrin, S., and Fava, R.A. (2005). Lymphotoxin-beta receptor signaling is required for the homeostatic control of HEV differentiation and function. Immunity *23*, 539–550.

Dejardin, E., Droin, N.M., Delhase, M., Haas, E., Cao, Y., Makris, C., Li, Z.W., Karin, M., Ware, C.F., and Green, D.R. (2002). The lymphotoxin-beta receptor induces different patterns of gene expression via two NF-kappaB pathways. Immunity *17*, 525–535.

Derudder, E., Dejardin, E., Pritchard, L.L., Green, D.R., Korner, M., and Baud, V. (2003). RelB/p50 dimers are differentially regulated by tumor necrosis factor-alpha and lymphotoxin-beta receptor activation: critical roles for p100. J. Biol. Chem. *278*, 23278–23284.

Ghosh, S., and Karin, M. (2002). Missing pieces in the NF-kappaB puzzle. Cell *109* (*Suppl*), S81–S96.

Hatada, E.N., Nieters, A., Wulczyn, F.G., Naumann, M., Meyer, R., Nucifora, G., McKeithan, T.W., and Scheidereit, C. (1992). The ankyrin repeat domains of the NF-kappa B precursor p105 and the protooncogene bcl-3 act as specific inhibitors of NF-kappa B DNA binding. Proc. Natl. Acad. Sci. USA *89*, 2489–2493.

Hoffmann, A., and Baltimore, D. (2006). Circuitry of nuclear factor kappaB signaling. Immunol. Rev. *210*, 171–186.

Hoffmann, A., Levchenko, A., Scott, M.L., and Baltimore, D. (2002). The IkappaB-NF-kappaB signaling module: temporal control and selective gene activation. Science *298*, 1241–1245.

Hoffmann, A., Leung, T.H., and Baltimore, D. (2003). Genetic analysis of NF-kappaB/Rel transcription factors defines functional specificities. EMBO J. *22*, 5530–5539.

Inoue, J., Kerr, L.D., Kakizuka, A., and Verma, I.M. (1992). I kappa B gamma, a 70 kd protein identical to the C-terminal half of p110 NF-kappa B: a new member of the I kappa B family. Cell *68*, 1109–1120.

Ishimaru, N., Kishimoto, H., Hayashi, Y., and Sprent, J. (2006). Regulation of naive T cell function by the NF-kappaB2 pathway. Nat. Immunol. *7*, 763–772.

Jiang, X., Takahashi, N., Matsui, N., Tetsuka, T., and Okamoto, T. (2003). The NF-kappa B activation in lymphotoxin beta receptor signaling depends on the phosphorylation of p65 at serine 536. J. Biol. Chem. *278*, 919–926.

Karin, M., Yamamoto, Y., and Wang, Q.M. (2004). The IKK NF-kappa B system: a treasure trove for drug development. Nat. Rev. Drug Discov. *3*, 17–26.

Katakai, T., Hara, T., Sugai, M., Gonda, H., and Shimizu, A. (2004). Lymph node fibroblastic reticular cells construct the stromal reticulum via contact with lymphocytes. J. Exp. Med. *200*, 783–795.

Kearns, J.D., Basak, S., Werner, S.L., Huang, C.S., and Hoffmann, A. (2006). I kappa B epsilon provides negative feedback to control NF-kappa B oscillations, signaling dynamics, and inflammatory gene expression. J. Cell Biol. *173*, 659–664.

Knuesel, M., Wan, Y., Xiao, Z., Holinger, E., Lowe, N., Wang, W., and Liu, X. (2003). Identification of novel protein-protein interactions using a versatile Mammalian tandem affinity purification expression system. Mol. Cell. Proteomics *2*, 1225–1233.

Krappmann, D., Emmerich, F., Kordes, U., Scharschmidt, E., Dorken, B., and Scheidereit, C. (1999). Molecular mechanisms of constitutive NF-kappaB/Rel activation in Hodgkin/Reed-Sternberg cells. Oncogene *18*, 943–953.

Lo, J.C., Basak, S., James, E.S., Quiambo, R.S., Kinsella, M.C., Alegre, M.L., Weih, F., Franzoso, G., Hoffmann, A., and Fu, Y.X. (2006). Coordination between NF-kappaB family members p50 and p52 is essential for mediating LTbetaR signals in the development and organization of secondary lymphoid tissues. Blood *107*, 1048–1055.

Mebius, R.E. (2003). Organogenesis of lymphoid tissues. Nat. Rev. Immunol. *3*, 292–303.

Mercurio, F., DiDonato, J.A., Rosette, C., and Karin, M. (1993). p105 and p98 precursor proteins play an active role in NF-kappa B-mediated signal transduction. Genes Dev. *7*, 705–718.

Moorthy, A.K., and Ghosh, G. (2003). p105.Ikappa Bgamma and prototypical Ikappa Bs use a similar mechanism to bind but a different mechanism to regulate the subcellular localization of NF-kappa B. J. Biol. Chem. *278*, 556–566.

Moorthy, A.K., Savinova, O.V., Ho, J.Q., Wang, V.Y., Vu, D., and Ghosh, G. (2006). The 20S proteasome processes NF-kappaB1 p105 into p50 in a translation-independent manner. EMBO J. *25*, 1945–1956.

Mordmuller, B., Krappmann, D., Esen, M., Wegener, E., and Scheidereit, C. (2003). Lymphotoxin and lipopolysaccharide induce NF-kappaB-p52 generation by a co-translational mechanism. EMBO Rep. *4*, 82–87.

Muller, J.R., and Siebenlist, U. (2003). Lymphotoxin beta receptor induces sequential activation of distinct NF-kappa B factors via separate signaling pathways. J. Biol. Chem. *278*, 12006–12012.

Naumann, M., Nieters, A., Hatada, E.N., and Scheidereit, C. (1993). NF-kappa B precursor p100 inhibits nuclear translocation and DNA binding of NF-kappa B/Rel-factors. Oncogene *8*, 2275–2281.

Ramakrishnan, P., Wang, W., and Wallach, D. (2004). Receptor-specific signaling for both the alternative and the canonical NF-kappaB activation pathways by NF-kappaB-inducing kinase. Immunity *21*, 477–489.

Rennert, P.D., James, D., Mackay, F., Browning, J.L., and Hochman, P.S. (1998). Lymph node genesis is induced by signaling through the lymphotoxin beta receptor. Immunity *9*, 71–79.

Schneider, K., Potter, K.G., and Ware, C.F. (2004). Lymphotoxin and LIGHT signaling pathways and target genes. Immunol. Rev. *202*, 49–66.

Senftleben, U., Cao, Y., Xiao, G., Greten, F.R., Krahn, G., Bonizzi, G., Chen, Y., Hu, Y., Fong, A., Sun, S.C., and Karin, M. (2001). Activation by IKKalpha of a second, evolutionary conserved, NF-kappa B signaling pathway. Science *293*, 1495–1499.

Shinkura, R., Kitada, K., Matsuda, F., Tashiro, K., Ikuta, K., Suzuki, M., Kogishi, K., Serikawa, T., and Honjo, T. (1999). Alymphoplasia is caused by a point mutation in the mouse gene encoding Nf-kappa B-inducing kinase. Nat. Genet. *22*, 74–77.

Tergaonkar, V., Correa, R.G., Ikawa, M., and Verma, I.M. (2005). Distinct roles of IkappaB proteins in regulating constitutive NF-kappaB activity. Nat. Cell Biol. *7*, 921–923.

Weih, D.S., Yilmaz, Z.B., and Weih, F. (2001). Essential role of RelB in germinal center and marginal zone formation and proper expression of homing chemokines. J. Immunol. *167*, 1909–1919.

Weih, F., and Caamano, J. (2003). Regulation of secondary lymphoid organ development by the nuclear factor-kappaB signal transduction pathway. Immunol. Rev. *195*, 91–105.

Werner, S.L., Barken, D., and Hoffmann, A. (2005). Stimulus specificity of gene expression programs determined by temporal control of IKK activity. Science *309*, 1857–1861.

Xiao, G., Harhaj, E.W., and Sun, S.C. (2001). NF-kappaB-inducing kinase regulates the processing of NF-kappaB2 p100. Mol. Cell *7*, 401–409.

Yamamoto, M., Yamazaki, S., Uematsu, S., Sato, S., Hemmi, H., Hoshino, K., Kaisho, T., Kuwata, H., Takeuchi, O., Takeshige, K., et al. (2004). Regulation of Toll/IL-1-receptor-mediated gene expression by the inducible nuclear protein IkappaBzeta. Nature *430*, 218–222.

Zarnegar, B., He, J.Q., Oganesyan, G., Hoffmann, A., Baltimore, D., and Cheng, G. (2004). Unique CD40-mediated biological program in B cell activation requires both type 1 and type 2 NF-kappaB activation pathways. Proc. Natl. Acad. Sci. USA *101*, 8108–8113.

Accession Numbers

The Matlab model is available upon request, and an SBML model version was submitted to http://www.ebi.ac.uk/biomodels and will be available with the identifier MODEL8478881246.