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Highlights

- Pickle selectively inhibits NF-κB target genes that are driven by Relish homodimers
- Pickle is a nuclear member of the IκB protein family
- Loss of *pickle* causes hyper-activation of Relish-dependent target genes
- Loss of *pickle* enhances host resistance to bacteria but compromises lifespan

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In Brief

Tight regulation of NF-κB signaling is critical to avoid detrimental and misbalanced responses. Morris et al. identify an IκB protein in *Drosophila* that inhibits a selective subset of the NF-κB dimer repertoire, thereby ensuring an appropriate immune response to pathogens while preventing tissue damage and reduced lifespan.

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Signal Integration by the IκB Protein Pickle Shapes Drosophila Innate Host Defense

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SUMMARY

Pattern recognition receptors are activated following infection and trigger transcriptional programs important for host defense. Tight regulation of NF-κB activation is critical to avoid detrimental and misbalanced responses. We describe Pickle, a Drosophila nuclear IκB that integrates signaling inputs from both the Imd and Toll pathways by skewing the transcriptional output of the NF-κB dimer repertoire. Pickle interacts with the NF-κB protein Relish and the histone deacetylase dHDAC1, selectively repressing Relish homodimers while leaving other NF-κB dimer combinations unscathed. Pickle’s ability to selectively inhibit Relish homodimer activity contributes to proper host immunity and organismal health. Although loss of pickle results in hyper-induction of Relish target genes and improved host resistance to pathogenic bacteria in the short term, chronic inactivation of pickle causes loss of immune tolerance and shortened lifespan. Pickle therefore allows balanced immune responses that protect from pathogenic microbes while permitting the establishment of beneficial commensal host-microbe relationships.

INTRODUCTION

Host defense against pathogen invasion relies on potent inflammatory responses that are controlled by the NF-κB family of transcription factors (Hayden and Ghosh, 2008). Activation of these transcription factors sets in motion a program aimed at clearing the pathogen. To restore homeostasis of the infected organ, such programs also induce modulators that, through negative feedback, regulate their temporal outputs to achieve balanced immune responses upon infection (Pasparakis, 2009).

NF-κB proteins share the presence of an N-terminal Rel homology domain (RHD), which is responsible for DNA binding as well as homo- and heterodimerization (Hayden and Ghosh, 2008). NF-κB proteins carry either an extended C-terminal stretch that contains multiple copies of ankyrin repeats (p105, p100, and Drosophila Relish) or a C-terminal transcription activation domain (κ-Rel, RelB, RelA [p65], and the Drosophila Dorsal [dD] and Dif [Dorsal-related immune factor] protein) (Gilmore, 2006). NF-κB dimers bind to κB sites within the promoters and enhancers of target genes and regulate transcription through the recruitment of coactivators and corepressors (Hayden and Ghosh, 2008). The combinatorial diversity of NF-κB homo- and heterodimers contributes to the regulation of distinct, but overlapping, transcriptional programs (Smale, 2012).

The activity of NF-κB is regulated by interaction with inhibitory IκB proteins (Gilmore, 2006). The IκB family proteins include, at least, eight dedicated IκB proteins: IκBα, IκBβ, IκBγ, IκBε, IκBβ, IκBNS, Bcl-3, and Drosophila Cactus. All IκB proteins harbor multiple ankyrin repeat regions (ARRs) through which IκBs bind to the RHDs of NF-κB dimers and regulate their transcriptional response. Generally, individual IκBs associate preferentially with a particular set of NF-κB dimers (Gilmore, 2006). Studying the function, mechanism of activation, and regulation of these factors is crucial for understanding host responses to microbial infections, immunological memory, and commensal-host interactions.

Drosophila can engage two pathways to activate NF-κB: the Toll pathway is activated primarily by fungal and Gram-positive infections, while the Immune deficiency (Imd) pathway responds mainly to Gram-negative infections (Buchon et al., 2014; Lemaitre et al., 1995, 1996).

Toll activation is triggered by Lys-type peptidoglycans (PGNs) as well certain bacterial virulence factors and components of fungal cell walls (El Chamy et al., 2008; Gottar et al., 2006; Michel et al., 2001). The Toll pathway initiates via an extracellular proteolytic cascade that culminates in the cleavage and activation of Spa(16) (Spz), which binds to the transmembrane Toll receptor and initiates an intracellular signaling cascade that results in the phosphorylation-dependent degradation of the IκB protein.
Cactus (Ganesan et al., 2011). This enables nuclear translocation of the NF-κB transcription factors Dif and dI (Lemaitre et al., 1996; Manfruelli et al., 1999; Rutschmann et al., 2002). Of these NF-κB proteins, Dif is the predominant transactivator in the antifungal and anti-Gram-positive bacterial defense in adults (Lemaitre et al., 1996; Manfruelli et al., 1999; Meng et al., 1999; Rutschmann et al., 2000a). Dorsal can substitute for Dif in larvae (Manfruelli et al., 1999; Rutschmann et al., 2000b).

The Imd pathway is activated by Gram-negative bacteria via two DAP-type PGN recognition receptors, plasma-membrane PGRP-LC and cytosolic PGRP-LE (Buchon et al., 2014). Binding of PGN to the receptors results in recruitment of an Ub-dependent signaling complex consisting of Imd, dFadd, and the caspase-8 homolog Dredd (Ganesan et al., 2011). Dredd is activated in an Ub-dependent manner with the help of the E3-ligase inhibitor of apoptosis 2 (Diap2) (Klein et al., 2005; Leulier et al., 2006; Meisander et al., 2012). Once active, Dredd cleaves off an inhibitory C-terminal ankyrin repeat of Relish, allowing translocation of the active RHD-containing N-terminal portion (RelN) to the nucleus, where it can act to induce activation of Relish-dependent target genes (Ganesan et al., 2011).

Activation of Toll and Imd pathways induces the expression of distinct but overlapping groups of NF-κB responsive antimicrobial peptide (AMP) genes, which are important for fending off invading microorganisms (Buchon et al., 2014). Because dI, Dif, and RelN readily form homo- as well as heterodimers, the transcriptional output of NF-κB can vary depending on dimer compositions and co-factor association (Bonney et al., 2014; Busse et al., 2007; Goto et al., 2008; Han and Ip, 1999; Tanji et al., 2007, 2010). How organisms are able to detect the presence of pathogens, and in response trigger balanced expression of innate defense genes, is a major question. It is clear that the expression repertoire and duration of immune defense genes must be tightly balanced to effectively clear pathogens while avoiding deleterious immune activation and tissue damage. Whereas pathogens frequently trigger multiple pattern recognition receptors, it remains unclear how these signals are integrated into an appropriate defense response to clear the pathogen. Here we report the identification and characterization of a Drosophila member of the IκB superfamily, which we term Pickle.

RESULTS

Pickle Negatively Regulates the NF-κB Transcription Factor Relish

To identify regulators of NF-κB signaling, we performed an in vitro RNAi mini-screen of proteins that interact with the Drosophila NF-κB protein Relish (Guruharsha et al., 2011; Rhee et al., 2014). This identified CG5118 as a putative negative regulator of Relish (Figure 1). In S2 cells, knockdown of CG5118, subsequently referred to as Pickle, caused hyperinduction of Imd-dependent AMP (AMP) genes following treatment with PGN from Gram-negative bacteria (Figures 1A, S1A, and S1B). Conversely, overexpression of Pickle strongly suppressed PGRP-LCx-, Imd-, and RelN-mediated induction of AMPs (Figures 1B–1D). This suggests that Pickle regulates the Imd pathway at the level of RelN. Accordingly, Pickle had no effect on Relish processing upon immune activation (Figure S1C). Whereas Pickle inhibited both Imd- and RelN-mediated production of AMPs, Pickle suppressed only PGRP-LCx- and Imd-induced activation of AMP genes.

The observation that Pickle suppresses RelN-driven induction of AMPs strongly suggests that Pickle directly regulates active, processed Relish. Consistently, we found that Pickle readily bound to the RelN portion of Relish (Figures 1E, 1F, and S1D), which is in agreement with previous proteomic-based studies (Guruharsha et al., 2011; Rhee et al., 2014). Detailed interaction analysis revealed that Pickle homo-oligomerizes (Figure S1E) and that the C-terminal half (aa 277–525) of Pickle was necessary and sufficient for RelN binding (Figure 1F). Although Pickle efficiently bound to Relish, it did not interact with other members of the Drosophila NF-κB family, such as dI and Dif (Figure S1D). Subcellular fractionation revealed that FLAG-tagged Pickle predominantly resides in the nuclear fraction (Figure 1G). Intriguingly, expression of Pickle appeared to sequester RelN in the nucleus, as significantly less RelN was present in the cytoplasmic fraction following co-expression with Pickle (Figure 1G).

The histone deacetylase dHDAC1 (also referred to as Rp3d) reportedly negatively regulates the transactivation of Relish (Kim et al., 2005, 2007), even though dHDAC1 does not directly bind to Relish (Kim et al., 2007). We therefore tested whether Pickle interacts with dHDAC1. We found that Pickle selectively co-purified endogenous dHDAC1 from cellular extracts (Figure 1H). Together, our data suggest that Pickle is a negative regulator of the Imd pathway that binds and inhibits the activity of the Relish, possibly via dHDAC1 recruitment.

Pickle Is a Member of the IκB Superfamily of Proteins

All currently known IκB proteins from vertebrates and invertebrates carry C-terminal ARRs with which they bind to the RHDs of NF-κB proteins (Hayden and Ghosh, 2008). Using sequence analysis and structural prediction algorithms, we identified seven ARRs within the C-terminal portion of Pickle (Figures 2A and S2), the portion that is necessary and sufficient for Relish binding. The N-terminal portion of Pickle did not harbor any recognizable motifs or domains. Because Pickle selectively binds to the RHD of Relish via its C-terminal ARRs and inhibits Relish activity, Pickle fulfills all functional and structural criteria of IκB proteins.

Phylogenetic analysis of Pickle with all currently known IκBs revealed that Pickle, along with its orthologs, is part of a clade of the IκB phylogenetic tree. IκB phylogenetic rooted tree reconstruction identified five major clades among the IκB proteins (Figure 2B). These major clades include (1) Pickle and Relish with NF-κB1 and NF-κB2 (53.4% bootstrap value), (2) Cactus with IκBz (61.3% bootstrap value), (3) IκBε (95.4% bootstrap value), (4) IκBβ (99.7% bootstrap value), and (5) Bcl-3 with IκBz and IκBNS (nuclear IκB proteins; 50.9% bootstrap value). The tree organization was validated using rooted and unrooted phylogenetic trees of invertebrate IκBs (Figure 2C). Pickle clustered along with Relish in both whole IκB and invertebrate-specific phylogenetic trees, with a bootstrap support of 100%. Our distance analysis demonstrates that pickle represents the direct arthropod homolog of the relish gene, albeit lacking a RHD in the N terminus and a PEST domain in its C terminus. Taken together, our functional, phylogenetic, and sequence analysis identifies Pickle, and its orthologs, as a member of the IκB superfamily.
Loss of pickle Results in Hyper-Activation of Relish Target Genes upon Infection

Next we investigated the role of Pickle in regulating Drosophila innate immune responses. Septic injury with the Gram-negative bacteria Erwinia carotovora carotovora 15 (Ecc15) resulted in hyper-activation of Imd signaling in flies in which pickle was knocked down in the fat body (Figures 3A and S3A). Although knockdown of pickle resulted in hyper-activation of Relish target genes, pickle inactivation did not affect Dif-mediated induction of Drosomycin following activation of the Toll pathway via septic injury with the Gram-positive, Lys-type PGN containing bacteria Micrococcus luteus (M.lut) (Figures S3C and S3D). Pickle, therefore, selectively modulates Imd signaling.

pickle also controlled the Imd pathway in the fly midgut following oral infection. Accordingly, feeding Gram-negative Ecc15 or Pseudomonas entomophila (P.e) caused upregulation of multiple Relish target genes in dissected midguts (Figures 3B and S3E). Compared with control flies, induction of Relish-dependent genes was significantly greater in flies with enterocyte-specific knockdown of pickle (Figures 3B, S3A, S3B, and S3E). pickleP[EPgy2]EY18569 null mutant flies (hereafter referred to as pickle+), which carry a transposon inserted 24 bp downstream of the translational start site of pickle (Figures 3C and S3G), also hyper-activated Relish target genes following systemic infection with Ecc15 (Figure 3D). Likewise, oral infection with Ecc15 or P.e similarly caused a dramatic over-production of Relish-dependent target genes (Figures 3E and 3Q). Essentially the same results were obtained using either homozygous pickle+ or pickle+Df1 flies that carry deletions of the pickle locus (Df1: Df[2L]Exel7006; Df2: Df[2L]BSC481) (Figures 3M and S3).
3D, 3E, 3G, and S3F). Of note, following systemic infection, Defensin induction was strongly reduced in homozygous pickle<sup>vy</sup> flies when compared to wild-type (WT) animals (yw and w<sup>1118</sup>). This effect is due to a background mutation in pickle<sup>vy</sup> flies because the reduced Defensin levels did not complement when pickle<sup>vy</sup> was placed trans-heterozygous over pickle-uncovering deficiency.

Figure 2. Phylogenetic Relationship of Pickle with Other IκB Family Members
(A) Schematic representation of Pickle (top) and its predicted 3D structure (middle). The predicted structure of the seven ARRs of Pickle (magenta) was superimposed onto the structure of Bcl-3 (PDB: 1K1A; cyan) (bottom).
(B) Phylogenetic analysis of IκB proteins. The sponge Amphimedon queenslandica was considered as an out-group. Bootstrap values > 50% have been provided. Members: IκBα (red), IκBβ (wine), IκBε (tan), Bcl-3 (brown), IκBNS (yellow), IκBζ (cyan), Cactus (dark green), Relish (orange), NF-κB1 (blue), NF-κB2 (light green), and Pickle (magenta).
(C) Phylogenetic relationship of Pickle with IκB family members present in invertebrates only using neighbor-joining method. Bcl-3 from Nematostella vectensis was considered as an outgroup (shown in black). Bootstrap scores > 60% have been provided. Members: Cactus (dark green), Relish (orange), NF-κB1 (blue), and Pickle (magenta).
See Table S1 for details. See also Figure S2.
alleles (Figures 3F and S3F). This background effect only affects the expression of Defensin, not other AMPs, and was observed following only systemic, not oral, infection (Figures 3D–3G and S3F). This is evident as oral infection with Ecc15 or P.e caused elevated Defensin levels in homozygous pickle

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The P[EPgy2] transposon in the pickle locus (EY18569) carries an upstream activating sequence element that permits GAL4-mediated re-expression of pickle commencing from an ATG at the end of the P[EPgy2] transposon (Belien et al., 2004). P[EPgy2] transposon-mediated re-expression generates Pickle lacking the eight N-terminal residues (Figure S3G). Because re-expression of Pickle rescues hyper-activation of AMPs in pickle

picklesuppresses spontaneous induction of Relish-dependent target genes in the absence of infection and maintains fly lifespan

For a host to tolerate a certain amount of resident bacteria, it is critical that the activation threshold of the immune response be tightly regulated (Buchon et al., 2014). Because pickle is a selective negative regulator of Relish, we investigated whether pickle contributes to the activation threshold of Relish-dependent target genes by suppressing Relish activity. Using the sterile environment of S2 cells, we found that mere knockdown of pickle led to a dramatic induction (>5,000-fold) of the basal levels of Dipterin A (DiptA) and Dipterin B (DiptB) (Figure 4A). Likewise, tissue-specific knockdown of pickle in the gut (enterocytes) or fat body led to a marked increase in the basal levels of pickle

Diptericin A

Diptericin B

Defensin
Figure 4. *pickle* Suppresses Spontaneous Induction of Relish-Dependent Target Genes in the Absence of Infection and Maintains Lifespan (A–E) qRT-PCR analysis of mRNAs of the indicated samples. (A) Relative AMP mRNA levels from unchallenged S2* cells following RNAi of the indicated genes. S2* cells treated for 4 hr with DAP-PGN are shown as reference point. (B) Relative AMP mRNA levels from unchallenged whole flies. RNAi knockdown was restricted to fat body (FB) cells using *c564::Gal4*. Relative AMP mRNA levels of control flies injected with *Ecc15* (2,000 CFU) (6 hr) served as reference point. (C) Relative AMP mRNA levels of dissected midguts from unchallenged female flies. RNAi knockdown was restricted to enterocytes (EC) using *myo::Gal4*. (legend continued on next page)
of AMP gene expression in unchallenged flies (Figures 4B and 4C). Transcript levels of AMP genes were also significantly elevated in dissected midguts of unchallenged pickle<sup>vy</sup> and trans-heterozygous pickle<sup>vy/DI</sup> and pickle<sup>vy/Df</sup> animals (Figure 4D). However, unlike in S2* cells, the elevated expression of AMPs in midguts of pickle<sup>vy</sup> flies was dependent on the presence of commensal bacteria, as this phenotype was lost when flies were reared under sterile conditions (Figure 4E). These data suggest that Pickle contributes to immune tolerance in the gut, preventing aberrant Relish-activity in response to gut microbiota. The difference between S2* cells and cells of the midgut may reflect cell- and tissue-type dependent differences.

Previous work indicated that chronic hyper-activation of Imd signaling in the gut reduces lifespan (Guo et al., 2014; Paredes et al., 2011). To test whether loss of pickle impacts on lifespan, we made use of the GeneSwitch system (Mathur et al., 2010), which negates genetic background effects (He and Jasper, 2014). Consistent with the notion that gut-specific knockdown of pickle results in hyper-activation of Imd signaling, we found that long-term, GeneSwitch-mediated depletion of pickle in enteroblasts and enterocytes caused a significant reduction in lifespan (Figures 4G–4I). Under the same conditions, GeneSwitch-mediated depletion of lacZ had no effect (Figures 4F and 4I). Together these data demonstrate that depletion of pickle results in hyper-activation of Imd signaling in the gut, which, similar to the loss of other Imd pathway negative regulators (Paredes et al., 2011), may compromise lifespan.

**Pickle Is Induced in Response to Commensal and Infectious Bacteria**

Expression of several negative regulators of the Imd pathway, such as pick and PGRP-LB, are regulated by Relish, allowing negative-feedback control of Imd signaling (Aggarwal et al., 2008; Kleino et al., 2008; Lhocine et al., 2008; Zaidman-Rémy et al., 2006). We found that pickle levels were significantly higher in midguts of conventionally reared (CR) animals than in germ free (GF) counterparts (Figure 5A). This indicates that pickle expression in the midgut is influenced by the presence of commensal bacteria, an observation that is consistent with a recent micro-array study (Broderick et al., 2014). Following oral infection, induction of pickle varied depending on the type of Gram-negative bacteria. Whereas oral infection with Ecc15 did not induce pickle expression (Figure 5B), exposure to the entomopathogenic bacteria P. aeruginosa caused a significant increase in pickle expression (Figure 5C). A similar bacteria-specific induction of pickle was also noted previously (Buchon et al., 2009a; Chakrabarti et al., 2012). Unlike pickle, expression of pick was induced in response to both these Gram-negative bacteria (Figures 5B–5D). Consistent with the notion that pickle and pick are regulated differently, we found that exposure to P. aeruginosa (Figures 5B–5D). Upon systemic infection, the induction of pickle is relatively modest (<2 times) (Figure S4A), which is in agreement with previous micro-array studies (De Gregorio et al., 2002; Irving et al., 2001). This was unlike pick, which was strongly upregulated in an Imd-dependent manner upon systemic infection (Figure S4B). Although the pathway or pathways that regulate pickle expression remain to be identified, pickle expression in the midgut appeared not to be induced by tissue damage per se (Figures S4C and S4D). Together, our data demonstrate that pickle is induced, albeit moderately, in response to commensal microbiota, and infection with certain types of bacteria.

**Pickle Selectively Inhibits RelN Homodimers**

The RHD of NF-κB proteins mediates DNA binding as well as homo- and heterodimerization (Hayden and Ghosh, 2008). In Drosophila, concomitant activation of the Toll and Imd pathways reportedly drives the formation of a complex network of Dif, dl, and Relish homo- and heterodimers (Tanji et al., 2010). Different dimer combinations are thought to activate overlapping transcriptional programs that vary in intensity, duration, and target genes (Smale, 2012). Because Pickle selectively binds to RelN (Figure 1), we tested the ability of Pickle to regulate various NF-κB homo- and heterodimer combinations. Whereas expression of Pickle strongly suppressed the transactivation ability of RelN as well as linked RelN<sup>+</sup>RelN homodimers (Figures 6A, 6F, 6G, 6H, S5A, and S5F; the caret represents the flexible peptide linker), Pickle failed to inhibit Dif, dl, and linked d<sup>+</sup>RelN or d<sup>+</sup>RelN dimer combinations (Figures 6B–6E and S6B–S6E). Of note, the ability of Pickle to repress induction of AttD and AttA was irrespective of the level of induction (Figures 1D and S5G–S5J). Intriguingly, the inability of Pickle to suppress linked Dif<sup>+</sup>RelN and dl<sup>+</sup>RelN was not due to lack of Pickle-binding, as Pickle readily co-purified Dif<sup>+</sup>RelN and dl<sup>+</sup>RelN from cellular extracts (Figure S5K). This suggests that Pickle requires two RelN moieties to inhibit transactivation.

Next, we investigated the impact of Pickle when both the Imd and Toll pathways are simultaneously activated in vivo. To that end, we used injection of heat-killed (hk) E. coli (E.coli) and M. lut and examined gene expression after 6 hr. Heat-killed bacteria were used to avoid any complication due to different bacterial growth rates. Interestingly, we found that loss of pickle (pickle<sup>vy</sup> and pickle<sup>vy/DI</sup>) hyper-activated AttD only when AttD was driven by RelN-only, such as following injection with E.coli (hk) (Figures 6G–6J, S6L, and S6M). In contrast, loss of pickle had no effect on AttD expression following co-injection of E.coli (hk) + M. lut (hk) (Figure 6H), a condition that induces AttD expression in an Imd- and Toll-dependent manner. This is entirely consistent with the notion that Pickle selectively inhibits target gene induction when such genes are exclusively

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(D) Analysis of flies with the indicated genotypes was conducted as in (C). (E) Relative AMP mRNA levels of dissected midguts from unchallenged female flies reared under conventional or axenic conditions.

(F–H) Lifespan experiments using the geneswitch system. Knockdown was restricted to enteroblasts (EBs)/enterocytes (ECs) using the geneswitch driver 5966:GS.

(i) Statistical summary of experiments shown in (F–H).

Histograms express results as percentage of a control sample (marked with dotted line). Unless otherwise indicated, p values were calculated from control using an unpaired Student’s t test. Results are representative of three (B–E) or two biological repeats (A). Mean ± SEM of biological (B–E) or experimental (A) repetitions.

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, and ****p ≤ 0.0001.
pickle Alters Host Resistance following Infection with Pathogenic Bacteria

To study the physiological relevance of Pickle in selectively inhibiting RelN homodimers, we examined the response of pickle mutants to infection with the pathogenic bacteria *L. monocytogenes* (*L.mono*) and *P. rettgeri* (*P.ret*). Six hours after infection, both these bacteria activated both the Imd and Toll pathways, some AMPs (*Defensin*) displayed different pathway dependency depending on the infecting bacteria. Induction of *AttD* in response to *L.mono* and *P.ret* infection was dependent solely on the Imd pathway (Figures 7A and 7B). *Defensin*, on the other hand, was solely Imd-dependent upon *L.mono* infection, whereas it was co-dependent on the Imd and Toll pathways following infection with *P.ret*. Interestingly, loss of *pickle* hyper-activated *AttD* and *Defensin* only when these AMPs were driven solely by RelN, such as following infection with *L.mono* (*AttD* and *Defensin*) and *P.ret* (*AttD*). Likewise, *c564::Gal4*-driven re-expression of *pickle* rescued the levels of *AttD* and *Defensin* expression to WT levels only when these AMPs were exclusively driven by RelN (Figure 7A). In contrast, loss of *pirk* caused hyper-activation of *AttD* and *Defensin* irrespective of the infecting bacteria, and irrespective of whether these AMPs were driven in an Imd- or Imd/Toll-dependent manner. Unlike *AttD* and *Defensin*, expression of *DiptA* and *DiptB* was insensitive to modulation by negative regulators such as *pirk* or *pickle*, quite possibly because these AMPs are already maximally induced. Our data are consistent with the notion that Pickle affects NF-κB target gene expression only when such genes are driven exclusively by RelN-only.

Next, we tested the ability of *pickle* to modulate the survival of flies infected with *L.mono*, *P.ret*, and *B. subtilis* (*B.sub*). *B.sub* is driven by RelN. Of note, the overall level of *AttD* induction did not influence the ability of *pickle* to regulate RelN-driven expression of *AttD*. This is evident as injection of live *Ecc15*, which drives *AttD* induction in a purely Imd-dependent manner, triggered the strongest upregulation of *AttD* (Figure 6I). Nevertheless, loss of *pickle* caused significant hyper-activation of *AttD*.

Overall, our data strongly suggest that Pickle selectively inhibits RelN homodimers, while leaving Dif:RelN heterodimers unscathed (Figure 6J). Of note, at present we cannot rule out the possibility that synergistic induction of AMPs is mediated by cooperating homodimers (Figure S5N), instead of heterodimers. Regardless of whether the *Drosophila* NF-κB proteins can act as either self-contained heterodimers or cooperating heterodimers, our data clearly demonstrate that Pickle only affects target gene expression when such genes are driven exclusively by RelN-only. As such, these data are entirely consistent with our observations using compound NF-κB dimers in S2* cells.
another pathogenic bacteria that activates both Imd and Toll pathways (Buchon et al., 2009b). Interestingly, pickle<sup>ey/Df1</sup> mutant flies were significantly less susceptible to systemic infection with <i>L.mono</i>, <i>P.ret</i>, and <i>B.sub</i> (Figures 7C, 7E, 7F, and S6C). In some instances, pickle appeared haploinsufficient, as pickle<sup>ey/+</sup> flies were significantly protected against <i>L.mono</i> and <i>P.ret</i> (~200 colony-forming units [CFU]) infection compared with WT animals (w<sup>1118</sup>). Notably, this was dependent on bacterial dose, as at a higher dose (~10,000 CFU), pickle<sup>ey/+</sup> and WT flies rapidly succumbed to <i>P.ret</i> infection, whereas pickle<sup>ey/Df1</sup> flies were significantly protected (Figure 7F). c564::Gal4-mediated re-expression of pickle in the fat body re-sensitized heterozygous flies to systemic bacterial infection (Figures 7C, 7E, and S6C), corroborating the specificity of the observed phenotype.

Figure 6. Pickle Selectively Inhibits RelN Hmodimers

(A–F) Relative AttD mRNA levels of S2* cells transiently transfected with plasmids expressing the indicated proteins. All proteins are FLAG-tagged at their N termini. Histograms depict mean ± SEM of three biological repeats. Results are expressed as percentage of induced GFP control samples in each experiment, and statistical significance is measured from these using an unpaired Student’s t test.

(G–I) Relative AttD levels mRNAs from unchallenged flies or flies injected with the indicated hk or live (Ecc15, 2,000 CFU) bacteria (6 hr). Unless otherwise indicated, statistical significance was measured from unchallenged w<sup>1118</sup> flies using an unpaired Student’s t test.

(J) Model depicting Pickle-mediated regulation of RelN.

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, and ****p ≤ 0.0001. See Figure S5.
The enhanced resistance of pickle^{ey/Df1} flies to *L. mono* was accompanied with a reduced bacterial load. Accordingly, pickle^{ey/Df1} flies harbored significantly fewer *L. mono* CFUs at 24 and 48 hr post-infection compared with WT controls (Figure 7D). Because *ref*^{220} or *imd*^{3} mutant flies are acutely sensitive to infection with *B. subtilis*, *L. mono*, or *P. rettgeri* (Figures S6D–S6F) (Buchon et al., 2009b; Mansfield et al., 2003), our data are consistent with a model whereby loss of *pickle* results in enhanced RelN-dependent immunity.

**DISCUSSION**

Tight regulation of NF-κB signaling is critical, as misbalanced and prolonged responses are detrimental to the host (Pasparakis, 2012). Here, we demonstrate that Pickle is required to prevent hyper-activation of Relish-dependent target genes. While loss of *pickle* improves host resistance to a variety of pathogenic bacteria, chronic inactivation of *pickle* compromises immune tolerance and shortens overall lifespan.

Pickle is a member of the IκB superfamily of proteins that selectively suppresses the production of Relish-dependent target genes. Like other IκB proteins, Pickle harbors C-terminal AARs through which it binds to the RHD of Relish and inactivates Relish-mediated target gene expression, possibly via the recruitment of the histone deacetylase dHdac1. Even though Pickle can bind to tethered Dif^ReIN and dÎ±^ReIN heterodimers, it suppresses NF-κB target gene expression only when such genes are driven solely by RelN. Accordingly, expression of Pickle strongly suppresses the trans-activation ability of RelN as well as RelN^ReIN homodimers (Figures 6). By contrast, Pickle fails to inhibit Dif, dl, and linked dÎ±^ReIN or Dif^ReIN dimer combinations. Moreover, under conditions in which the Toll and Imd pathways are simultaneously activated, *pickle* exclusively influences induction of AMPs that are driven by RelN-only (Figures 6 and 7). Pickle, therefore, likely “skews” the output of both pathways via selective inhibition of genes solely transactivated by Relish. This is unlike Pirk, which...
regulates pathway flux, and does not selectively inhibit a specific subset of the NF-κB dimer repertoire.

Although homo- and heterodimers mediate diverse effects in mammalian systems (Hayden and Ghosh, 2008), it has been suggested that in Drosophila NF-κB proteins might mediate their effects as cooperating homodimers bound to distinct κB sites, rather than as heterodimers bound to a single site (Busse et al., 2007). Despite good evidence to suggest that heterodimers function in Drosophila (Han and Ip, 1999; Senger et al., 2004; Tanji et al., 2010), we cannot rule out the possibility that synergistic induction of AMPs is mediated by cooperating homodimers. Regardless of whether the Drosophila NF-κB proteins can act as either self-contained heterodimers or cooperating homodimers, our data demonstrate that Pickle inhibits AMP induction only when RelN is the only NF-κB member driving target gene expression. Under conditions in which AMPs are driven cooperatively by Dif and RelN, or Dif and dl, AMP production is insensitive to the presence of Pickle.

Pickle’s ability to bias the output of certain Relish-dependent target genes, namely, those that are driven solely by RelN:RelN, has important physiological consequences. In the short term, loss of pickle enhances expression of RelN target genes, significantly boosting the host defense from infection with pathogenic bacteria. Although we observed elevated levels of several AMPs in pickle mutant flies, mere hyper-activation of these AMPs was not the only reason these animals were protected. pick mutant animals similarly hyper-activated these AMP genes, yet these animals were unable to fend off L.mono, P.ret, and B.sub. The difference between loss of pickle and loss of pick is likely due to the differential regulation of Imd signaling. Because Pirk regulates Imd signaling at the level of the receptor (or Imd) (Aggarwal et al., 2008; Kleino et al., 2008; Lhocine et al., 2008), Pirk is unable to skew the Imd and Toll signaling outputs toward a subset of NF-κB target genes that are driven by a particular NF-κB dimer combination. Although in the short term, loss of pickle appears to be beneficial for immune defense against certain pathogenic bacteria (L.mono, P.ret, and B.sub), in the long run, chronic inactivation of pickle results in loss of immune tolerance and shortened lifespan. Pickle, therefore, allows for a balanced immune response that protects from pathogenic microbes while permitting the establishment of beneficial commensal host-microbe relationships. At present little is known how the host tolerates commensal bacteria while mounting a full response to others. Our observations are consistent with a model in which Pickle acts as an immune modulator that balances the complex relationship between host resistance to pathogens and immune tolerance to microbiota. Because breakdown of this balance contributes to the development of immune-related pathologies (Pasparakis, 2009), further dissection of Pickle’s unique regulatory action may aid our understanding of how aberrant NF-κB activity contributes to dysfunction of the immune system.

**EXPERIMENTAL PROCEDURES**

**Fly Stocks, Husbandry, and Bacterial Cultures**

Flies were kept at 25 °C, unless stated otherwise. A full list of all genotypes used for each figure can be found in Table S2. Bacterial cultures were initiated from single colonies grown on LB plates. Small volumes of the starter cultures were then diluted at least 1:1,000 (so as to have an near undetectable optical density [OD]) and cultured up to the desired OD on the day of the experiment. For hk M.lut and E.coli solutions, bacteria were suspended in sterile PBS and subsequently hk for 10 min at 95 °C in a heating block. Heat-killed bacterial solutions were diluted so as to enable the injection of approximately equal numbers of E.coli (hk) and M.lut (hk). Preparations were then aliquoted and frozen at −80 °C for repeat use of identical hk bacterial preparations. See Supplemental Experimental Procedures for details.

**Systemic Infection Experiments and Survival**

Three- to eight-day-old adult flies were used for infection experiments. Systemic infection was performed by injecting flies with 13.8 nl of a cultured bacterial solution, PBS, or hk bacteria resuspended in PBS, using the Nanject II (Drummond Scientific). Flies were then incubated at 25 °C, transferred to fresh vials every day, and collected and examined at different time points for qRT-PCR, CFU counts, and survival analysis.

**Oral Infection and Bleomycin Treatments and Generation of Axenic Flies**

Oral infections and treatments were performed as previously reported (Buchon et al., 2009a), with some modifications. Briefly, 5- to 7-day-old female flies were raised, starved, and fed on a Whatman filter paper covered by 150 μl of an infection solution (Ecc15 at OD 100 or P. and OD 50) or 250 μg/ml bleomycin solution (Sigma) containing 2.5% sucrose. See Supplemental Experimental Procedures for details.

**Generation of Axenic Flies**

Freshly laid eggs (≤5 hr old) were collected from grape juice agar plates. Embryos were rinsed in 1 x PBS, and any hatched larvae or loose agar pieces were removed with sterile forceps. All subsequent steps were performed in a sterilized laminar flow hood. Embryos were surface-sterilized with 70% ethanol and then by 5% sodium hypochlorite for 10 min, followed by three washes with sterile water, and then aseptically transferred to sterile food in a small amount of 100% ethanol. Adult female flies (about 7 days old) were collected for midgut dissection.

**Lifespan Analysis**

Five virgins 5966:GS homozygotes were crossed to one male with the indicated genotypes. Ten crosses were set up per genotype. Progeny were collected and allowed to mate for 2 days. Male siblings were then separated (20 flies per vial). Flies were treated with RU486, as previously described (Guo et al., 2014), with some modifications. See Supplemental Experimental Procedures for details.

**Bacterial Load**

The bacterial load was established as previously described (Khallil et al., 2015). Fly homogenates were serially diluted (10-fold), and CFUs were counted manually. Ten flies were analyzed per genotype and experimental repeat. A “mock” procedure lacking injected bacteria was performed in each experiment repeat. No CFUs were detectable following this “mock” procedure.

**qRT-PCR and Primer Sequences**

qRT-PCR was performed as previously described (Meinander et al., 2012), with some modifications. For whole-fly analysis in Figures 3, 4, and S3, pools of 15 male and 15 female flies per sample were analyzed. For whole-fly analysis in Figures 6, 7, S5, and S6, pools of 5 female flies per sample were analyzed. For midgut analysis, pools of 15–20 dissected female midguts were analyzed. The amount of mRNA detected was normalized to control rp49 mRNA values. In Figures 5, 6, S4, and S5, the ΔCtΔΔCt/ΔCtΔΔCt ratios are indicated to allow comparison of the actual expression levels. For the remaining figures, relative ΔCtΔΔCt/ΔCtΔΔCt ratios of WT controls were set at 100%, and the fold differences were calculated using the ΔΔCt method. See Supplemental Experimental Procedures for additional details and primer sequences.

**Tissue Culture and Treatments**

Drosophila S2* cells were a kind gift from Neal Silverman. S2* cells were cultured at 23 °C in Schneider’s Drosophila medium (Gibco), supplemented with 10% fetal bovine serum, 60 μg/ml penicillin, and 100 μg/ml streptomycin. RNAi knockdown was performed as described previously (http://www.flyrnai.)
org/DRSC-PRR.html). Transfections were performed using Effectene (Qiagen) or calcium phosphate protocol (Clontech) according to the manufacturer’s instructions. See Supplemental Experimental Procedures for details.

Immunoprecipitation, Nuclear and Cytoplasmic Fractionation, and Western Blot Analysis
Immunoprecipitation and western blot analysis were performed as previously described (Meinander et al., 2012), with some modifications. Cytoplasmic and nuclear fractions were separated via combined use of centrifugation and cytoplasmic and nuclear extraction buffers. See Supplemental Experimental Procedures for details.

Sequence Collection, Phylogenetic Analysis, and Model Constructions
Analysis was performed as previously described (Basith et al., 2013). The 3D model of Pickle was built using ANK-N5C (Protein Data Bank [PDB]: 4O60) as a template, which shares a sequence identity of 26.8%. See Supplemental Experimental Procedures for details.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, six figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2016.08.003.

AUTHOR CONTRIBUTIONS
O.M. designed and performed the experiments. X.L. and N.B. conducted and supervised the experiments involving Drosophila midgut. C.D. provided advice throughout systemic infection experiments. C.R. performed preliminary experiments investigating the immune function of pickle. T.T. gave advice throughout western blotting experiments. A.C. and G.P. performed preliminary experiments investigating the pathological impact of pickle. H.C. performed preliminary experiments involving Drosophila midgut. S.B. and S.C. performed bioinformatics analysis. O.M. and P.M. designed the study and wrote the manuscript.

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