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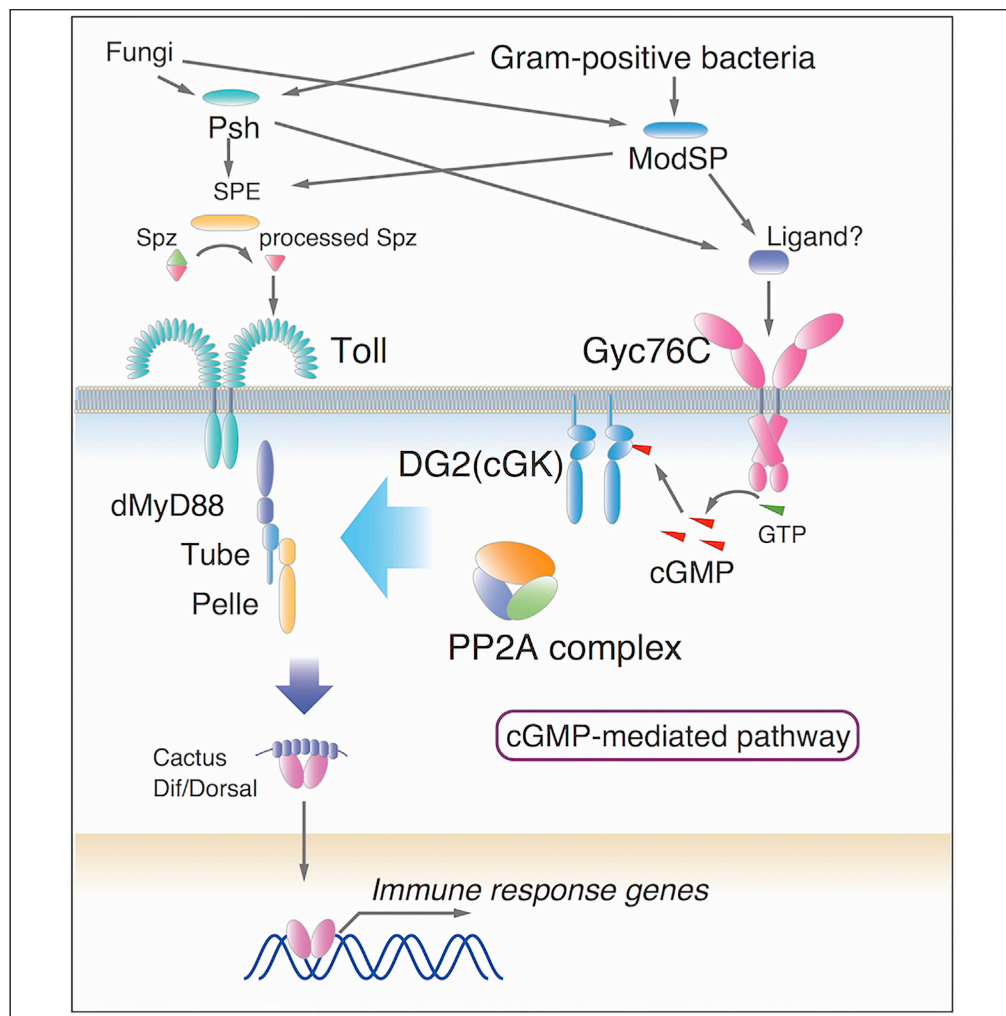

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Highlights

Drosophila NF-κB signaling is activated by Gyc76C in parallel with the Toll receptor

Gyc76C modulates NF-κB signaling through downstream Toll receptor components

In *Drosophila*, the pathway comprises a cGMP-dependent protein kinase (cGK) and PP2A

In human cells, a membrane-bound cGK, PRKG2, also modulates NF-κB signaling via PP2A

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cGMP signaling pathway that modulates NF-κB activation in innate immune responses

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SUMMARY

The nuclear factor-kappa B (NF-κB) pathway is an evolutionarily conserved signaling pathway that plays a central role in immune responses and inflammation. Here, we show that *Drosophila* NF-κB signaling is activated via a pathway in parallel with the Toll receptor by receptor-type guanylate cyclase, Gyc76C. Gyc76C produces cyclic guanosine monophosphate (cGMP) and modulates NF-κB signaling through the downstream Tollreceptor components dMyd88, Pelle, Tube, and Dif/Dorsal (NF-κB). The cGMP signaling pathway comprises a membrane-localized cGMP-dependent protein kinase (cGK) called DG2 and protein phosphatase 2A (PP2A) and is crucial for host survival against Gram-positive bacterial infections in *Drosophila*. A membrane-bound cGK, PRKG2, also modulates NF-κB activation via PP2A in human cells, indicating that modulation of NF-κB activation in innate immunity by the cGMP signaling pathway is evolutionarily conserved.

INTRODUCTION

The nuclear factor-kappa B (NF-κB) signaling pathway plays a central regulatory role in a wide range of biologic processes, including innate and adaptive immune responses and inflammation (Hayden and Ghosh, 2004; Vallabhapurapu and Karin, 2009). A key feature of the innate immune response—an evolutionarily conserved barrier to infectious pathogens (Hoffmann and Reichhart, 2002) extensively investigated in the fruit fly *Drosophila melanogaster* (Buchon et al., 2014; Hoffmann and Reichhart, 2002)—is the rapid induction of antimicrobial peptides (AMPs) in the *Drosophila* fat body, the functional equivalent of the mammalian liver (Buchon et al., 2014). Two distinct NF-κB signaling pathways, the Toll and immune deficiency (IMD) pathways, control the induction of AMPs and are mechanistically similar to the mammalian NF-κB signaling pathways, the Toll-like receptor (TLR)/interleukin-1 (IL-1) receptor signaling pathway and the tumor necrosis factor alpha receptor signaling pathway, respectively (Buchon et al., 2014; Hoffmann and Reichhart, 2002). The Toll pathway is mainly involved in immune defense against Gram-positive bacterial and fungal infections (Lemaitre et al., 1996). Peptidoglycan recognition protein (PGRP)-SA and Gram-negative bacteria-binding protein 1 (GNBP-1) recognize the lysine-type peptidoglycans of Gram-positive bacteria, whereas GNBP-3 recognizes the β-glucans of fungi. Both sets of recognition proteins trigger the activation of modular serine protease (ModSP) (Buchon et al., 2009; Chamy et al., 2008; Gobert et al., 2003; Gottar et al., 2006), which then activates the serine protease cascade. This cascade activates the Spätzle-processing enzyme (SPE), which cleaves the cytokine-like protein Spätzle (Spz) (Buchon et al., 2009; Jang et al., 2006; Weber et al., 2003). SPE is also activated by some bacterial and fungal proteases through Persephone (Psh) (Ligoxygakis et al., 2002; Gottar et al., 2006; Chamy et al., 2008; Issa et al., 2018). Processed Spz binds to the Toll receptor, which then recruits an adaptor protein, *Drosophila* myeloid differentiation primary response 88 (dMyd88) (Tauszig-Delamasure et al., 2002; Wasserman, 2000; Weber et al., 2003). The serine threonine kinase Pelle is then recruited by the adaptor protein Tube to form a heterotrimeric complex (dMyd88-Tube-Pelle) (Daigneault et al., 2013; Sun et al., 2004; Towb et al., 1998). This complex regulates the phosphorylation and degradation of Cactus, a *Drosophila* inhibitor κB (IκB, an NF-κB inhibitory protein), leading to the activation and nuclear localization of Dif and Dorsal (*Drosophila* NF-κB factors) (Belvin et al., 1995; Ip et al., 1993; Manfruelli et al., 1999; Reach et al., 1996). The TLR signaling

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pathway, which regulates innate immune responses in mammals (Medzhitov et al., 1997; Takeuchi and Akira, 2010), is mediated by two distinct pathways through distinct adaptor proteins, MyD88 and Toll/IL-1-receptor-domain-containing adaptor inducing interferon-β (TRIF) (Takeuchi and Akira, 2010). Similar to the *Drosophila* Toll pathway, IL-1-receptor-associated kinase (IRAK) 4 and IRAK 1/2, counterparts of *Drosophila* Tube and Pelle, mediate MyD88-dependent TLR signaling, leading to the phosphorylation and degradation of IκB (Kawagoe et al., 2008; Lin et al., 2010). The IMD pathway is mainly involved in immune defense against Gram-negative bacterial infections and is mediated by several other factors, including PGRP-LE and PGRP-LC receptors, Imd adaptor protein, and Relish (another NF-κB factor in *Drosophila*) (Buchon et al., 2014; Gottar et al., 2002; Takehana et al., 2002; Choe et al., 2002; Rämet et al., 2002; Georgel et al., 2001; Stoven et al., 2003). The IMD pathway is also regulated by nitric oxide (Foley and O'Farrell, 2003), which can activate the cyclic guanosine monophosphate (cGMP) signaling pathway.

Guanylate cyclase (GC) converts guanosine triphosphate (GTP) into cGMP, which acts as a second messenger to regulate a wide range of physiologic processes (Feil and Kemp-Harper, 2006). GCs consist of soluble (Montfort et al., 2017) and receptor-type (r)GCs (Kuhn, 2016) and are regulated by diverse extracellular ligands, such as peptide hormones, bacterial toxins, and free radicals, as well as by calcium and adenine nucleotides. Once GCs are activated, cGMP accumulates in the cytoplasm and initiates signaling cascades via downstream effectors, such as cGMP-dependent protein kinase (cGK), cGMP-regulated phosphodiesterases (PDE), and cyclic nucleotide-gated ion channels (Cng) (Tsai and Kass, 2009). cGMP signaling, which is evolutionarily conserved, is involved in renal homeostasis (Davies, 2006), behavior (Morton, 2011; Reaume and Sokolowski, 2009), development (Patel et al., 2012), and stress tolerance (Davies et al., 2014) in mammals and *Drosophila*. rGCs also function in inflammation and immune cell differentiation and regulation (Zhang et al., 2015). Despite the widespread roles of cGMP and rGCs, however, it is unclear whether rGC-dependent signaling and NF-κB signaling interact in the context of immune regulation.

Here, we report the existence of a cGMP-dependent signaling pathway that modulates NF-κB activation in *Drosophila* immune responses. This pathway was discovered through the identification of Gyc76C, an rGC that functions as an immune receptor. This Gyc76C-induced cGMP signaling pathway is mediated by the membrane-localized cGK called DG2 and by protein phosphatase 2A (PP2A). Our findings also show that *Gyc76C* and *dg2* (*foraging*) are crucial for host survival against Gram-positive bacterial infections in *Drosophila*. We also show that the membrane-bound cGK PRKG2 modulates NF-κB activation via PP2A in human cells as well, indicating an evolutionarily conserved role for this cGMP signaling pathway in modulating NF-κB activation in innate immunity.

RESULTS

A receptor guanylate cyclase, Gyc76C, activates NF-κB signaling (the Toll pathway) in parallel with the Toll receptor

Our previously reported genome-wide gain-of-function genetic screen based on modular misexpression using GAL4/UAS in *Drosophila* was used to identify immune-response activating genes (Momiuchi et al., 2015; Takehana et al., 2002; Yano et al., 2008). Here, we expanded this earlier screen with 11,761 additional lines and identified 8 lines that constitutively express two AMP reporter genes, *Drosomycin* (*Drs*)-*GFP* and *Diptericin* (*Dpt*)-*lacZ*, which monitor Toll and IMD pathway activation, respectively (Jung et al., 2001), in a GAL4-dependent manner. The GAL4/UAS system revealed the upregulated expression of *CG3221*, *CG3216*, and *CG10543*, which lie adjacent to a single UAS vector insertion in one of these eight lines, GS 2198 (Figures S1A and S1B). These three genes encode dim γ-tubulin 3, a putative rGC, and a putative zinc finger nucleic-acid-binding factor, respectively. We investigated the role of *CG3216* in immune responses, as *CG3216* encodes a putative receptor with an extracellular ligand-binding domain, a transmembrane domain, as well as intracellular kinase homology and guanylate cyclase domains; it also has amino acid sequence similarity with mammalian rGCs that produce cGMP, such as the atrial natriuretic peptide receptor (Davies, 2006).

Overexpression of *CG3216* in the fat body using the *c564*-GAL4 driver and in epithelial tissues such as the trachea using the *NP2610*-GAL4 driver resulted in the constitutive expression of *Drs-GFP* and *Dpt-lacZ* in the larval fat body and epithelia, respectively (Figure S1C). Quantitative RT-PCR analyses revealed that *CG3216* overexpression induces strong *Drs* expression and weak *Dpt* expression compared with the overexpression of positive regulators of the Toll pathway (processed Spz, Spz[C106], a Toll receptor ligand)

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(Lemaitre et al., 1996; Weber et al., 2003) and the IMD pathway (PGRP-LC, an IMD pathway receptor) (Gottar et al., 2002), indicating that *CG3216* overexpression preferentially activates the Toll pathway (Figure S1D). The rGC gene family in *Drosophila* includes *CG3216*, *Gyc76C*, *Gyc32E*, and *CG31183* (FlyBase data; http://flybase.org). Our analysis of *Drosophila* rGC expression in wild-type larvae revealed little endogenous expression of *CG3216* (Figure S1E) but high (Figure S1E), preferential expression of *Gyc76C* in immune-related tissues such as the fat body, hemocytes, and Malpighian (renal) tubules (Figure S1F, and FlyAtlas data; http://flyatlas.org/). Gyc76C also more effectively induced *Drs* expression than CG3216 (Figure S1G). *Gyc76C* overexpression also induced expression of *Drs-GFP* in larvae (Figure S1H). Gyc76C has known roles in neuronal pathfinding and development (Ayoob et al., 2004; Chak and Kolodkin, 2014) in muscle (Patel et al., 2012), epithelia (Myat and Patel, 2016), and wing (Schleede and Blair, 2015); salt stress response (Overend et al., 2012); and gut immunocompetence (Bou Sleiman et al., 2015). The present findings indicate that Gyc76C also has a role in the immune response.

Because *Gyc76C* overexpression preferentially induces the expression of *Drs* relative to *Dpt* (Figure 1A), we examined the relationship between Gyc76C and Toll pathway components by epistatic analysis in larvae. The *Gyc76C*-dependent induction of *Drs* was not affected by mutations of *Toll*, *spz*, or *imd* but was significantly suppressed by mutations of downstream components of the Toll pathway, Tube, Pelle, dMyd88, and Dif/Dorsal (NF-κB factors in the Toll pathway) (Figure 1B). In reciprocal experiments, processed Spz-dependent induction of *Drs* was not affected by a hypomorphic mutation caused by a P-element insertion in the first intron of *Gyc76C* ($gyc76C^{KG03723}$) (Ayoob et al., 2004) (Figure 1B). The *imd*-, *PGRP-LC*-, and *PGRP-LE*-dependent induction of *Dpt* and *Drs* was not affected in the presence of $gyc76C^{KG03723}$ (Figure S2A). Together, these findings indicate that Gyc76C induces *Drs* expression via downstream Toll receptor components in a Toll-receptor-independent manner. Consistent with these results, co-expression of processed Spz and *Gyc76C* led to a strong synergistic induction of *Drs* (Figure 1C). Epistasis in adults was not analyzed because *Gyc76C* expression in the fat body, as driven by *c564*-GAL4 (Figure S2B), and in the fat body and hemocytes, as driven by *Cg*-GAL4 (data not shown), failed to induce *Drs* when using a temperature-sensitive GAL80 that avoids the pupal lethality caused by *Gyc76C* expression. Consistent with these results, induction of *Gyc76C* mRNA expression and cGMP production by *Gyc76C* overexpression induced by *c564*-GAL4 was low in adults in comparison with larvae (Figures S2C and S2D). The production of cGMP is required for Gyc76C-dependent induction of *Drs*, as described below.

All rGC family members have a highly conserved cyclase domain that converts GTP to cGMP (Davies, 2006). *Gyc76C* overexpression induces *Drs* expression and cGMP production, and both constitutive *Drs* expression and cGMP production were severely reduced by a Gyc76C point mutation at amino acid 945 ($Gyc76C^{D945A}$) that disrupts cyclase activity (Ayoob et al., 2004) (Figure 1D). *Gyc76C*-dependent *Drs* induction was also severely reduced by the co-expression of *Drosophila* PDE-5/6, a cGMP-specific PDE that catalyzes the hydrolysis of cGMP to inactive 5′ nucleotides (Day et al., 2005) (Figure 1E). As expected, *PDE5/6* abolished the *Gyc76C*-induced increase in cGMP but did not affect the forced expression of *Gyc76C* driven by *c564*-GAL4 (Figures 1E and S2E). cGMP production was not induced by Toll pathway activation via processed Spz and activated Toll ($Toll^{10B}$) or by IMD pathway activation via *imd* and *PGRP-LC* (Figure S2F). Consistently, the expression of *PDE5/6*, which reduces cGMP levels, did not suppress the processed Spz-dependent induction of *Drs* or the *PGRP-LC*-dependent induction of *Dpt* (Figure S2G). Together, these findings demonstrate that a cGMP-mediated signaling pathway leads to AMP induction via downstream Toll receptor components that function in parallel with the Toll receptor.

Gyc76C mediates ModSP-dependent and psh-dependent NF-κB signaling activation in cooperation with the Toll receptor

As previously reported (Buchon et al., 2009), *Drs* is induced by the overexpression of ModSP, an upstream regulator of the Toll receptor (Figure 2A). The ModSP-dependent induction of *Drs* was stronger than that induced in response to the expression of Gyc76C, $Toll^{10B}$, or processed Spz (Figure 2A) but was suppressed by mutations of $gyc76C^{KG03723}$ and $Toll^{1\text{-}RXA/r632}$ (Figure 2B). Thus, the ModSP-dependent induction of *Drs* requires Gyc76C. This requirement was confirmed by a transheterozygote of $gyc76C^{KG03723}$ and *Df(3L) Exel9061* (Figure 2B). Forced expression of *ModSP* via *c564*-GAL4 was not affected by these mutations (Figure S2H). These findings indicate that both Gyc76C and the Toll receptor are required for ModSP-dependent induction of *Drs*, consistent with the synergistic induction of *Drs* by both cGMP pathway and Toll receptor activation resulting from the co-expression of Gyc76C and processed Spz (Figure 1C). GNBP-1 acts as a recognition protein that activates ModSP. GNBP-1-dependent induction of *Drs* was also suppressed

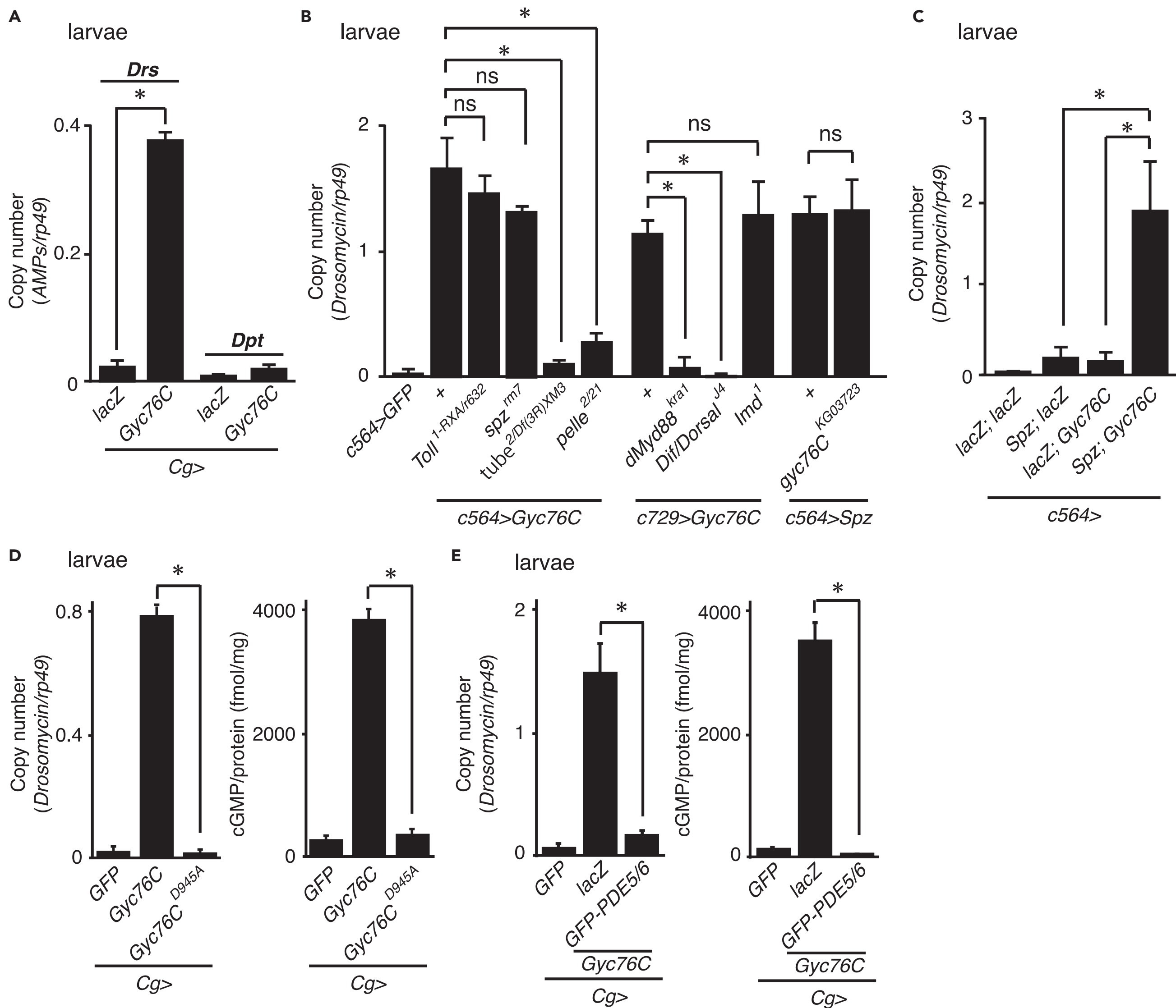

Figure 1. Gyc76C mediates the induction of antimicrobial peptides via the cGMP-mediated pathway involving downstream components of the Toll receptor

(A) *Gyc76C*-mediated induction of endogenous *Drs* and *Dpt* by quantitative RT-PCR. *Gyc76C* or *lacZ* were induced by *Cg*-GAL4.

(B) *Gyc76C* expressed in different Toll pathway mutant backgrounds, $Toll^{1\text{-}RXA/r632}$, spz^{rm7}, $tube^{2/Df(3R)XM3}$, $pelle^{2/21}$, $dMyd88^{kra1}$, and $Dif/Dorsal^{J4}$; in an IMD pathway mutant background, imd^{1}; and in a control background, +. *Drs* expression in larvae was measured by quantitative RT-PCR. In reciprocal experiments, processed Spz (Spz[C106]) was expressed in $gyc76C^{KG03723}$ background larvae. *Gyc76C* expression was induced by *c564*-GAL4 or *c729*-GAL4.

(C) Synergistic induction of *Drs* by the overexpression of *Gyc76C* and processed Spz in larvae.

(D) $Gyc76C^{D945A}$ is a cyclase-defective mutant. Its effects on *Drs* expression and cGMP levels in larvae are shown.

(E) PDE5/6 is a cGMP-degrading enzyme. Its effects on *Gyc76C*-mediated *Drs* expression and cGMP production in larvae are shown. $^{*}P < 0.05$, ns (not significant) $P > 0.1$, Student's t test.

Data are represented as mean, and error bars indicate standard deviation. Data shown are representative of three independent experiments.

by the $gyc76C^{KG03723}$ mutation, providing further support that Gyc76C mediates ModSP-dependent NF-κB activation (Figure 2C). To investigate whether Gyc76C ligand activity is induced in larvae by *ModSP* overexpression, we used the *Drs* reporter (*Drs*-luciferase) in *Drosophila* DL1 cells in which *Gyc76C* is expressed (Figure S2J). The *Drs* expression was induced by treating DL1 cells with an acid (0.1% trifluoroacetic acid in H_2O) extract of the homogenate of larvae expressing *lacZ* (Figure S2I). The *Drs*-inducing activity in the larval extract was increased by *ModSP* overexpression, which was reduced by RNAi targeting *Gyc76C* in DL1 cells (Figure S2I). Consistent with the partial reduction of *Drs*-inducing activity by *Gyc76C* RNAi, *Gyc76C* mRNA

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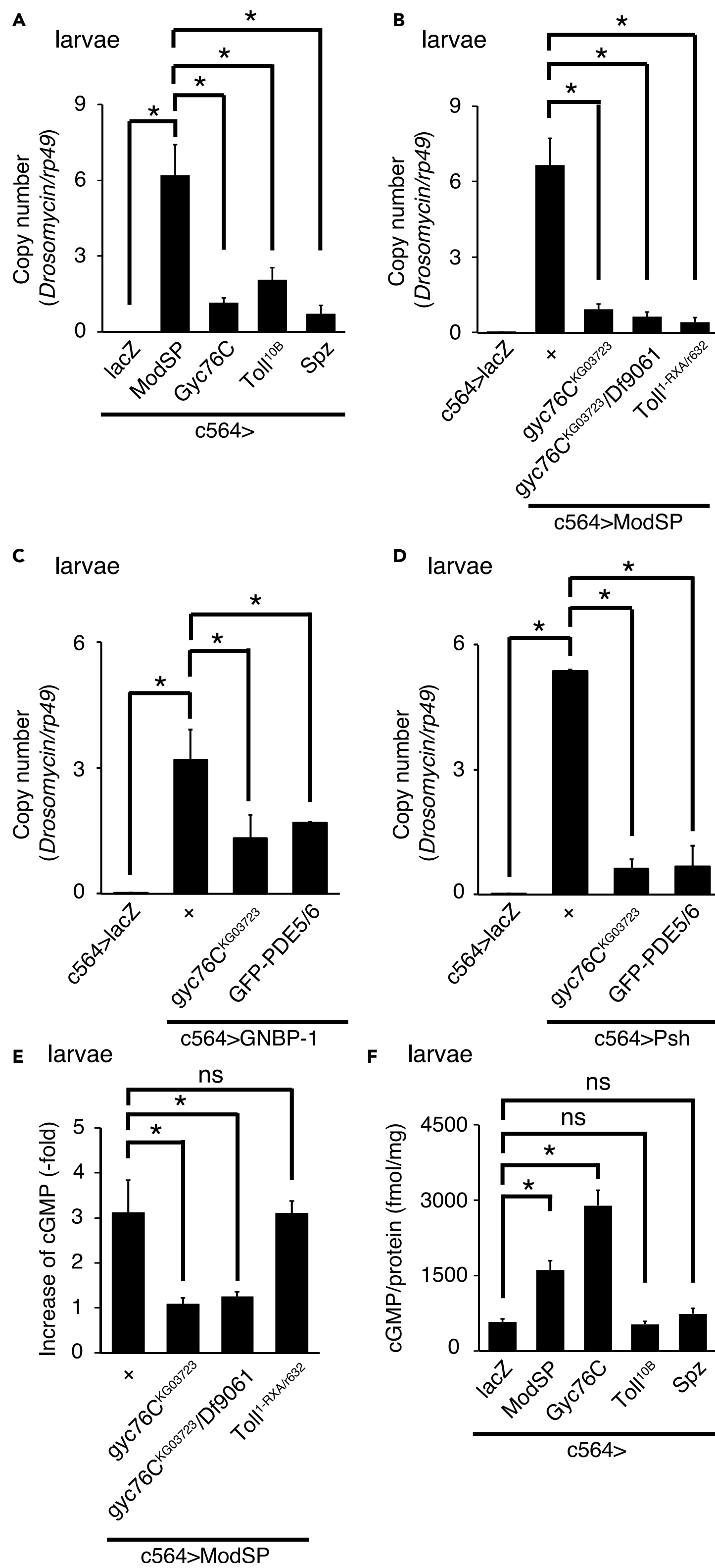

Figure 2. *Drs* expression is induced by independent activation of Gyc76C and the Toll receptor as a result of ModSP forced expression

(A) *Drs* induction by forced expression of ModSP, Gyc76C, activated Toll receptor ($Toll^{10B}$), and by processed Spz in larvae.

(B) *Gyc76C* and *Toll* mutations affect the ModSP-dependent induction of *Drs* in larvae.

(C) Effects of *Gyc76C* mutation and forced expression of PDE5/6 on the GNBP-1-dependent *Drs* expression.

(D) Effects of *Gyc76C* mutation and forced expression of PDE5/6 on the Psh-dependent *Drs* expression.

(E) Effects of *Gyc76C* and *Toll* mutations on the ModSP-dependent increase in cGMP levels in larvae. The ModSP-dependent cGMP increase is shown relative to that in *lacZ*-expressing larvae with the same genetic background. +: control (no mutation).

(F) Effects of forced expression of ModSP, Gyc76C, $Toll^{10B}$, and processed Spz on cGMP levels in larvae. $^{*}P < 0.05$, ns $P > 0.1$, Student's t test.

Data are represented as mean, and error bars indicate standard deviation. Data shown are representative of three independent experiments.

expression was partially reduced to ~50% in DL1 cells by *Gyc76C* RNAi (Figure S2J). These results suggest that the Gyc76C ligand exists in larvae overexpressing *ModSP*. The Gyc76C ligand activity was both acid- (0.1% trifluoroacetic acid) and heat- (100°C, 1 min) stable and was retained on the ultrafiltration membrane with a molecular weight cut-off of 10,000 (Millipore, Amicon Ultra-4 10K), suggesting that the Gyc76C ligand is an acid- and heat-stable molecule with a molecular weight greater than 10 kD (Table S1). The Toll pathway is activated by some bacterial and fungal proteases through Psh, independent of ModSP. As previously reported (Jang et al., 2006), *Drs* is induced by overexpression of *Psh* (Figure 2D). The Psh-dependent induction of *Drs* was suppressed by the $gyc76C^{KG03723}$ mutation (Figure 2D), suggesting that Gyc76C also mediates Psh-dependent NF-κB activation.

cGMP levels were also increased by *ModSP* overexpression (Figure 2E), a response that was suppressed by the $gyc76C^{KG03723}$, but not the $Toll^{1\text{-}RXA/r632}$, mutation (Figure 2E). Thus, Gyc76C is required for the ModSP-dependent increase in cGMP. This requirement was confirmed by analyzing the transheterozygote of $gyc76C^{KG03723}$ and *Df(3L)Exel9061* (Figure 2E). *Drs* was induced by $Toll^{10B}$ expression and processed Spz expression (Figure 2A), whereas cGMP was not (Figures 2F and S2F). These results indicate that NF-κB signaling activation is induced by the independent activation of Gyc76C and the Toll receptor through the forced expression of *ModSP*. GNBP-1-dependent and Psh-dependent *Drs* induction was reduced by co-expression with *PDE-5/6*, which reduces cGMP levels, confirming that cGMP production is required for GNBP-1-dependent and Psh-dependent *Drs* induction (Figures 2C and 2D).

Roles of the cGMP pathway and Gyc76C in resistance to Gram-positive bacterial infections

We next performed infection experiments to evaluate the role of *Gyc76C* and cGMP signaling in host defense against various pathogens. Because the *Gyc76C* null mutant is lethal (Ayoob et al., 2004), we used hypomorphic *Gyc76C* mutant flies, $gyc76C^{KG03723}$, in which endogenous *Gyc76C* mRNA expression is reduced to ~35% than in *yw* control flies (Figure S3A). In contrast to wild-type (Oregon-R) and w^{1118} flies, $gyc76C^{KG03723}$ flies were susceptible to Gram-positive bacterial infections (*Staphylococcus saprophyticus*, *Enterococcus faecalis*, and *Staphylococcus aureus*), but not to Gram-negative bacterial infections (*Erwinia carotovora carotovora 15* [*Ecc15*] and *Escherichia coli*), indicating an important role for *Gyc76C* in self-defense against Gram-positive bacteria (Figure 3A). This finding is consistent with the activation of Gyc76c by ModSP, which itself is activated by the recognition of Gram-positive bacteria (Buchon et al., 2009). Notably, in revertant flies in which the P element was precisely excised from the $gyc76C^{KG03723}$ genome, the *S. saprophyticus*-susceptible phenotype was restored, ruling out the possibility of genetic background effects (Figure 3A). The $gyc76C^{KG03723}$ flies accumulated significantly higher Gram-positive bacterial loads (Figure S3B), but not Gram-negative bacterial loads (Figure S3C), in their hemolymph relative to control *yw* flies. Susceptibility to *S. saprophyticus* infection was also induced by *Cg*-GAL4-mediated RNAi knockdown of *Gyc76C* in the fat body and hemocytes but not in *lacZ*-expressing control flies (Figures 3B and S3A). Susceptibility to Gram-positive bacterial infection was also more severe in flies in which both *Gyc76C* and *Toll* were knocked down by RNAi, relative to the susceptibility induced by RNAi-mediated targeting of *Toll* alone (Figure 3B). Expression of *PDE5/6* by *Cg*-GAL4 also induced susceptibility to Gram-positive bacterial infections, suggesting an important role for cGMP signaling in self-defense against Gram-positive bacteria (Figure 3C). A role for *Gyc76C* and cGMP signaling in host defense against Gram-positive bacteria was confirmed by larval infection experiments. In contrast to *yw* larvae, $gyc76C^{KG03723}$ larvae were susceptible to *S. saprophyticus* but not to *Ecc15* infection (Figure S4A).

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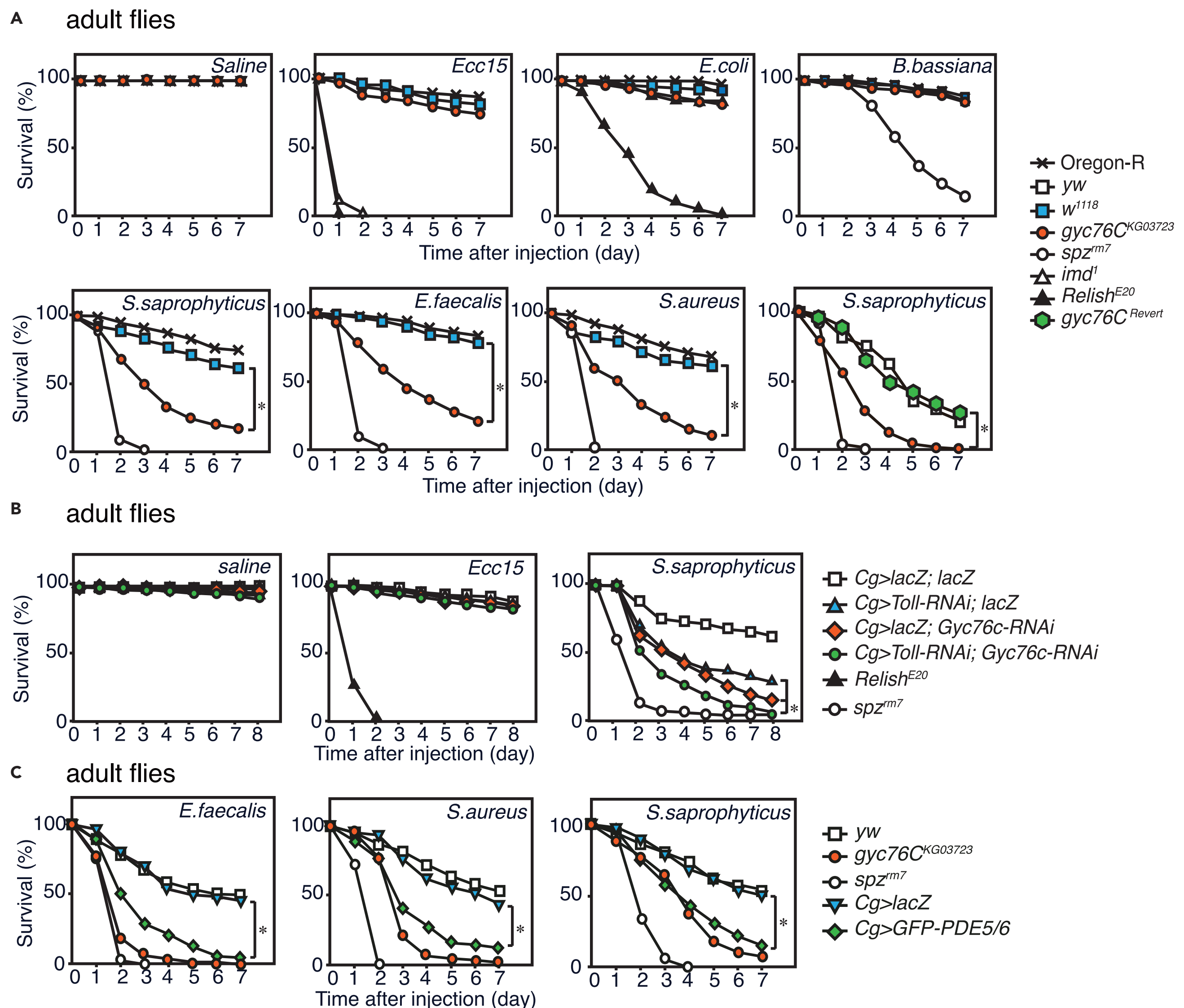

Figure 3. *Gyc76C* is required for host defense against Gram-positive bacterial infection in *Drosophila*

(A) Survival rate of control (Oregon-R, w^{1118}, and *yw*), $gyc76C^{KG03723}$, spz^{rm7}, imd^{1}, and $Relish^{E20}$ flies was assessed following the injection of saline (as a control), Gram-negative bacteria (*Ecc15* and *E. coli*), Gram-positive bacteria (*S. saprophyticus*, *E. faecalis*, and *S. aureus*), or after natural infection with *B. bassiana* at 28°C. Revertant flies of $gyc76C^{KG03723}$, $gyc76C^{Revert}$, were also infected with *S. saprophyticus*.

(B) Effects of RNAi knockdown of *Gyc76C*, *Toll*, or both *Gyc76C* and *Toll*, on survival following *S. saprophyticus*, *Ecc15*, and control (saline) infections.

(C) Effects of *PDE5/6* expression by a *Cg*-GAL4 driver on survival following *E. faecalis*, *S. aureus*, and *S. saprophyticus* infections. *lacZ* expression was used as a control. $^{*}P < 0.05$, Log rank test.

Data shown are represented as means of at least three independent experiments.

Expression of *Gyc76C* RNAi and of *PDE5/6* by *c564*-GAL4 also induced larval susceptibility to *S. saprophyticus* infections (Figures S4B and S4C). Susceptibility of $gyc76C^{KG03723}$ larvae to *S. saprophyticus* was completely rescued by *c564*-GAL4-dependent expression of *Gyc76C*, but not $Gyc76C^{D945A}$, which failed to produce cGMP (Figures S4D and 1D).

Interestingly, as previously reported (Lemaitre et al., 1996), the spz^{rm7} mutant was susceptible to natural fungal infection (*Beauveria bassiana*), whereas the $gyc76C^{KG03723}$ mutant was not, indicating a distinct role for Spz/Toll and Gyc76C in antifungal defense in *Drosophila* (Figure 3A). In addition to the natural infection experiments, we investigated the role of Gyc76C in host defense against fungal infection by injection in the same manner as for bacterial infection. Double mutant adults and larvae of *modSP* and

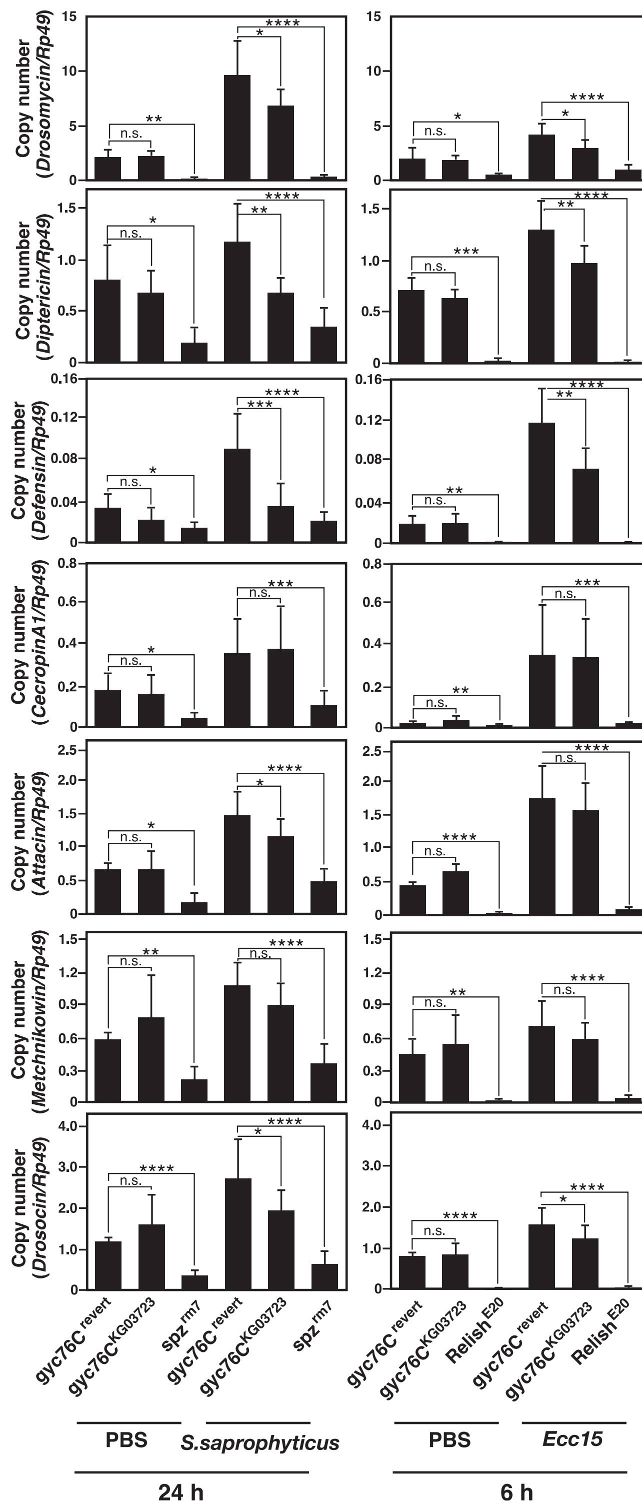

Figure 4. Antimicrobial peptide gene expression in mutant flies following Gram-positive and Gram-negative bacterial infections

At 24 h after *S. saprophyticus* injection or 6 h after *Ecc15* injection, the expression of seven AMP genes was measured in *gyc76C*^{KG03723}, *spz*^{rm7}, and *Relish*^{E20} flies, with the P-element excised line of *gyc76C*^{Revert} used as a control. To evaluate the infection-dependent induction of AMPs, phosphate-buffered saline (PBS) was used as a negative control. Data shown

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Figure 4. ***Continued***

are the means of at least three independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, n.s. (not significant), Student's t test.

Data are represented as mean, and error bars indicate standard deviation.

psh were susceptible to *Candida glabrata* injection, whereas *gyc76C*^{KG03723} mutant adults and larvae were not, indicating that Gyc76C-mediated NF-κB activation does not have a significant role in host defense against fungi, unlike its role in host defense against Gram-positive bacteria (Figures S4E and S4F).

Gyc76C is involved in inducing numerous genes against Gram-positive bacterial infections

To investigate the effect of the *Gyc76C* mutation on AMP induction, we compared AMP induction in *gyc76C*^{KG03723} and *Gyc76C*-revertant flies following Gram-positive and Gram-negative bacterial infections. *Drs* induction in response to *S. saprophyticus* infection was partially reduced in *gyc76C*^{KG03723} flies relative to that in *spz*^{rm7} flies, whereas the induction of *Defensin* (*Def*), an AMP that acts on Gram-positive bacteria, was reduced in both *gyc76C*^{KG03723} and *spz*^{rm7} flies following *S. saprophyticus* infection (Figure 4). The induction of *Dpt*, *Attacin*, and *Drosocin* was also reduced in *gyc76C*^{KG03723} flies after *S. saprophyticus* infection (Figure 4). Induction of *Def* in response to *Ecc15* infection was also reduced in *gyc76C*^{KG03723}, but, compared with *Relish*^{E20}, significant induction of the tested *AMPs* was observed in *gyc76C*^{KG03723} in response to *Ecc15* infection (Figure 4). DNA microarray analysis also revealed that the induction of 139 other genes was reduced in *gyc76C*^{KG03723} flies compared with controls following *S. saprophyticus* infection, including 9 genes that encode innate immune-related factors, mainly AMPs: 19 serine protease genes; 8 metallopeptidase genes; 5 polysaccharide-binding protein genes; and 4 metal-ion-binding protein genes (Figures S5A, S5B, and Table S1, for details see http://www.pharm.tohoku.ac.jp/~seimei/m_array/index.htm). Many of these genes were upregulated by *Gyc76C* overexpression in larvae, although they were suppressed in response to *S. saprophyticus* infection in *spz*^{rm7} flies (Table S1). DNA microarray analysis of *gyc76C*^{KG03723} larvae also confirmed that *Gyc76C* is involved in the induction of numerous genes, including innate immune-related genes, serine protease genes, and polysaccharide-binding protein genes, in response to Gram-positive bacterial infections in larvae (Table S1 and Figure S5C). Many of these genes were upregulated by *Gyc76C* overexpression in larvae and were suppressed in response to *S. saprophyticus* infection in Dif/Dorsal (*Drosophila* NF-κB) mutant larvae and in *psh;modSP* double mutant larvae (Table S1). Together, these findings demonstrate that *Gyc76C* is involved in the induction of numerous genes in response to Gram-positive bacterial infections.

The cGMP signaling pathway is mediated by the membrane-localized cGMP-dependent protein kinase DG2 and protein phosphatase 2A

cGK and Cng are candidate downstream mediators of cGMP signaling (Davies, 2006). We therefore investigated the effect of their loss on *Gyc76C*-dependent induction of *Drs* by RNAi-mediated knockdown of the *Drosophila* cGKs *dg2* (*foraging*), *dg1* (*Pkg21D*), and *CG4839* (*Pkg-like*) and two *Drosophila* Cngs, *Cng* and *Cng-like*. When *dg2* was knocked down in the fat body via *c564*-GAL4, *Gyc76C*-dependent induction of *Drs* was inhibited (Figure 5A). In contrast, RNAi-mediated knockdown of *Cng*, *Cng-like*, and *CG4839* in the fat body did not inhibit *Gyc76C*-dependent induction of *Drs*, whereas *dg1* RNAi induced embryonic lethality (data not shown). These results indicate that DG2, a membrane-localized cGK (Davies, 2006), mediates cGMP signaling, thus activating immune responses mediated by Gyc76C. RNAi-mediated knockdown of *dg2* by *Cg*-GAL4 in flies and by *c564*-GAL4 in larvae induced susceptibility to *S. saprophyticus* infection but not to *Ecc15* infection (Figures 5B and S4B). Susceptibility to *S. saprophyticus* infection was also induced by adult-stage-specific expression of *dg2* RNAi via *c564*-GAL4 using a temperature-sensitive GAL80, indicating that the *S. saprophyticus*-susceptible phenotype was not caused by developmental defects in the fat body (Figure S4G). These findings indicate that immune responses mediated by DG2 are crucial for self-defense against Gram-positive bacteria in *Drosophila* flies and larvae.

Consistent with the synergistic induction of *Drs* by co-overexpression of Gyc76C and processed Spz in larvae (Figure 1C), *Drs* was synergistically induced by co-overexpression of one of two isoforms of *dg2* (MacPherson et al., 2004), *P1* or *P2*, and processed Spz in larvae (Figure 5C). *Drs* and a *Drs* reporter gene (*Drs-luciferase*) were similarly synergistically induced by co-overexpression in *Drosophila* DL1 cells of *dg2* and *dMyd88* and that of *dg2* and an active Toll receptor (delta leucine-rich repeat) (Figures 5D and 5E). *Drs-luciferase* activation by the co-expression of *dg2* and *dMyd88* in DL1 cells was enhanced by *Gyc76C*, indicating that DG2 cooperates with Gyc76C to enhance dMyd88-dependent Dif/Dorsal

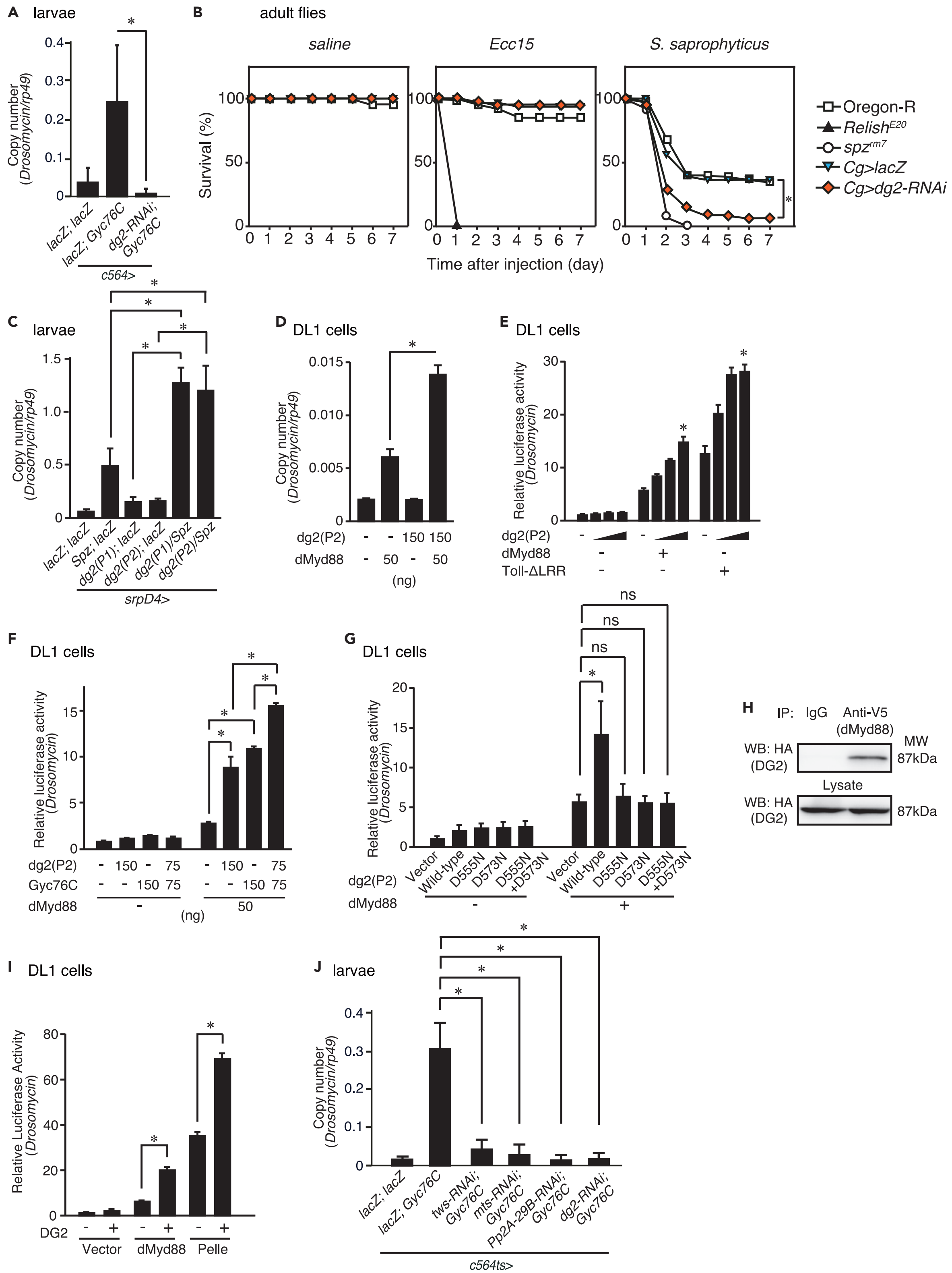
A larvae
Copy number (Drosomycin/rp49)
lacZ; lacZ
lacZ; Gyc76C
dg2-RNAi; Gyc76C
c564>
B adult flies
saline
Ecc15
S. saprophyticus
Survival (%)
Time after injection (day)
Oregon-R
Relish^E20
spz^rm7
Cg>lacZ
Cg>dg2-RNAi
C larvae
Copy number (Drosomycin/rp49)
lacZ; lacZ
Spz; lacZ
dg2(P1); lacZ
dg2(P2); lacZ
dg2(P1)/Spz
dg2(P2)/Spz
srpD4>
D DL1 cells
Copy number (Drosomycin/rp49)
dg2(P2)
dMyd88
(ng)
E DL1 cells
Relative luciferase activity (Drosomycin)
dg2(P2)
dMyd88
Toll-ΔLRR
F DL1 cells
Relative luciferase activity (Drosomycin)
dg2(P2)
Gyc76C
dMyd88
(ng)
G DL1 cells
Relative luciferase activity (Drosomycin)
dg2(P2)
dMyd88
Vector
Wild-type
D555N
D573N
D555N +D573N
ns
H
IP: IgG Anti-V5 (dMyd88)
WB: HA (DG2)
MW
87kDa
Lysate
I DL1 cells
Relative Luciferase Activity (Drosomycin)
DG2
Vector
dMyd88
Pelle
J larvae
Copy number (Drosomycin/rp49)
lacZ; lacZ
lacZ; Gyc76C
tws-RNAi; Gyc76C
mts-RNAi; Gyc76C
Pp2A-29B-RNAi; Gyc76C
dg2-RNAi; Gyc76C
c564ts>

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Figure 5. Membrane-localized cGMP-dependent protein kinase, DG2, and protein phosphatase 2A are involved in modulating NF-κB activation downstream of Gyc76C in *Drosophila*

(A) *Gyc76C*-mediated *Drs* induction in larvae following RNAi-mediated knockdown of *dg2*. $^*P < 0.05$, Student's t test.

(B) Survival of control (Oregon-R, *lacZ*-expressing), *dg2* RNAi-targeted using *Cg*-GAL4, spz^{rm7}, and $Relish^{E20}$ flies was assessed following the injection of saline (control), Gram-negative bacteria (*Ecc15*), and Gram-positive bacteria (*S. saprophyticus*) at 28°C. $^*P < 0.05$, Log rank test.

(C–E) Enhanced *Drs* (C and D) and *Drs-luciferase* (E) induction by processed Spz (C), dMyd88 (D and E), or active Toll receptor (delta leucine-rich repeat (E) and by the overexpression of *dg2* in larvae (C) and *Drosophila* DL1 cells (D and E).

(F) DG2 and Gyc76C co-operate in the enhancement of dMyd88-dependent Dif/Dorsal (*Drosophila* NF-κB) activation.

(G) Effects of kinase-defective DG2, D555N, and D573N on dMyd88-dependent Dif/Dorsal activation.

(H) Co-immune precipitation of DG2 with dMyd88. V5-tagged dMyd88 and HA-tagged DG2 were expressed in DL1 cells, and immune precipitation and Western blotting were performed using anti-V5 and anti-HA antibodies, respectively. IgG was used as a control.

(I) *Dg2* overexpression enhances Pelle-dependent induction of *Drs-luciferase* in *Drosophila* DL1 cells.

(J) RNAi-mediated targeting of *Drosophila* protein phosphatase 2A (PP2A) subunits, *tws*, *mts*, and *Pp2A-29B* affect *Gyc76C*-mediated *Drs* induction in larvae. $^*P < 0.05$, ns $P > 0.1$, Student's t test.

Data are represented as mean, and error bars indicate standard deviation. Data shown are representative of at least three independent experiments.

(*Drosophila* NF-κB) activation (Figure 5F). The enhanced activation of dMyd88-dependent Dif/Dorsal by *dg2* was abolished by point mutations in DG2 at amino acid 555 or 573 that disrupt cGMP-dependent kinase activity (Figure 5G), indicating that this DG2 kinase activity is required to enhance dMyd88-dependent Dif/Dorsal activation. Consistent with functional interactions of dMyd88 and DG2, co-immune precipitation results revealed that dMyd88 forms a complex with DG2 in DL1 cells (Figure 5H).

dMyd88 contains a consensus sequence for phosphorylation by cGK (RRXS/T) (Wong et al., 2012). Enhanced dMyd88-dependent Dif/Dorsal activation by DG2 was not reduced by a point mutation in this consensus sequence at amino acid 461 (S461A; Figure S6A). Moreover, a point mutation at amino acid 461 to glutamic acid (S461E) did not enhance dMyd88-dependent Dif/Dorsal activation (Figure S6A). These findings indicate that dMyd88 consensus sequence phosphorylation does not contribute to the dMyd88-dependent enhanced activation of Dif/Dorsal by DG2. In addition to dMyd88, Pelle-dependent Dif/Dorsal activation was also enhanced by DG2 (Figure 5I). The dMyd88-dependent enhanced activation of Dif/Dorsal by DG2 was suppressed by RNAi knockdown of *dMyd88*, *tube*, and *pelle* (Figure S6B). On the other hand, enhanced Pelle-dependent activation of Dif/Dorsal by DG2 was suppressed by *pelle* RNAi, but not by *dMyd88* and *tube* RNAi, indicating that *dMyd88* and *tube* are not required for DG2-induced enhancement of Pelle-dependent Dif/Dorsal activation (Figure S6C). Pelle does not contain a consensus sequence for cGK phosphorylation. These findings suggest that additional components exist between DG2 and Pelle in cGMP signaling.

To identify the genes involved in the modulation of NF-κB activation by DG2, we performed a genome-wide RNAi screen in DL1 cultured cells. The results revealed that the enhanced activation of dMyd88-dependent Dif/Dorsal by Gyc76C and DG2 was suppressed by RNAi-mediated knockdown of *pelle* and *Dif* (Figure S6D). We compared the results of this screen with those of a similar RNAi screen of dMyd88-dependent Dif/Dorsal activation (Kanoh et al., 2015) and identified *microtubule star* (*mts*, *Drosophila* PP2A catalytic subunit), *Pp2A-29B* (*Drosophila* PP2A regulatory subunit A), *twins* (*tws*, *Drosophila* PP2A regulatory subunit B), and *Pp4-19C* (*Drosophila* protein phosphatase 4 catalytic subunit), in addition to 22 other genes (Figures S6E and S6F). We investigated the effects of knocking down the *Drosophila* PP2A subunits *tws*, *mts*, and *Pp2A-29B* on the *Gyc76C*-dependent induction of *Drs* in larvae. The findings demonstrated that, similarly to when *dg2* was knocked down in the fat body by *c564*-GAL4 using a temperature-sensitive GAL80, loss of the *Drosophila* PP2A subunits inhibited the *Gyc76C*-dependent induction of *Drs* (Figure 5J). These findings indicate that both DG2 and PP2A modulate NF-κB activation by the cGMP pathway in *Drosophila*.

cGMP signaling modulation of NF-κB activation is conserved

Seven mouse and human rGC homologs, including the atrial natriuretic peptide receptor, are reported, but only one membrane-bound cGK (cGK II) is reported (Davies, 2006). Expression of the human cGK II, PRKG2, enhanced the MyD88-dependent activation of NF-κB signaling in human HEK293 cells, as well as *Drosophila* DL1 cells (Figure 6A). This enhanced MyD88-dependent NF-κB activation by PRKG2 was reduced by a PRKG2 point mutation at amino acid 576 that disrupts its cGMP-dependent kinase activity (Figure 6B). Thus, cGMP-dependent kinase activity of PRKG2 is required to enhance MyD88-dependent NF-κB activation in human cells, as it is in *Drosophila*. Deletion of the cGMP-binding domain of PRKG2 reduced this enhanced MyD88-dependent NF-κB activation by PRKG2 (Figure 6B). IRAK1 is the human

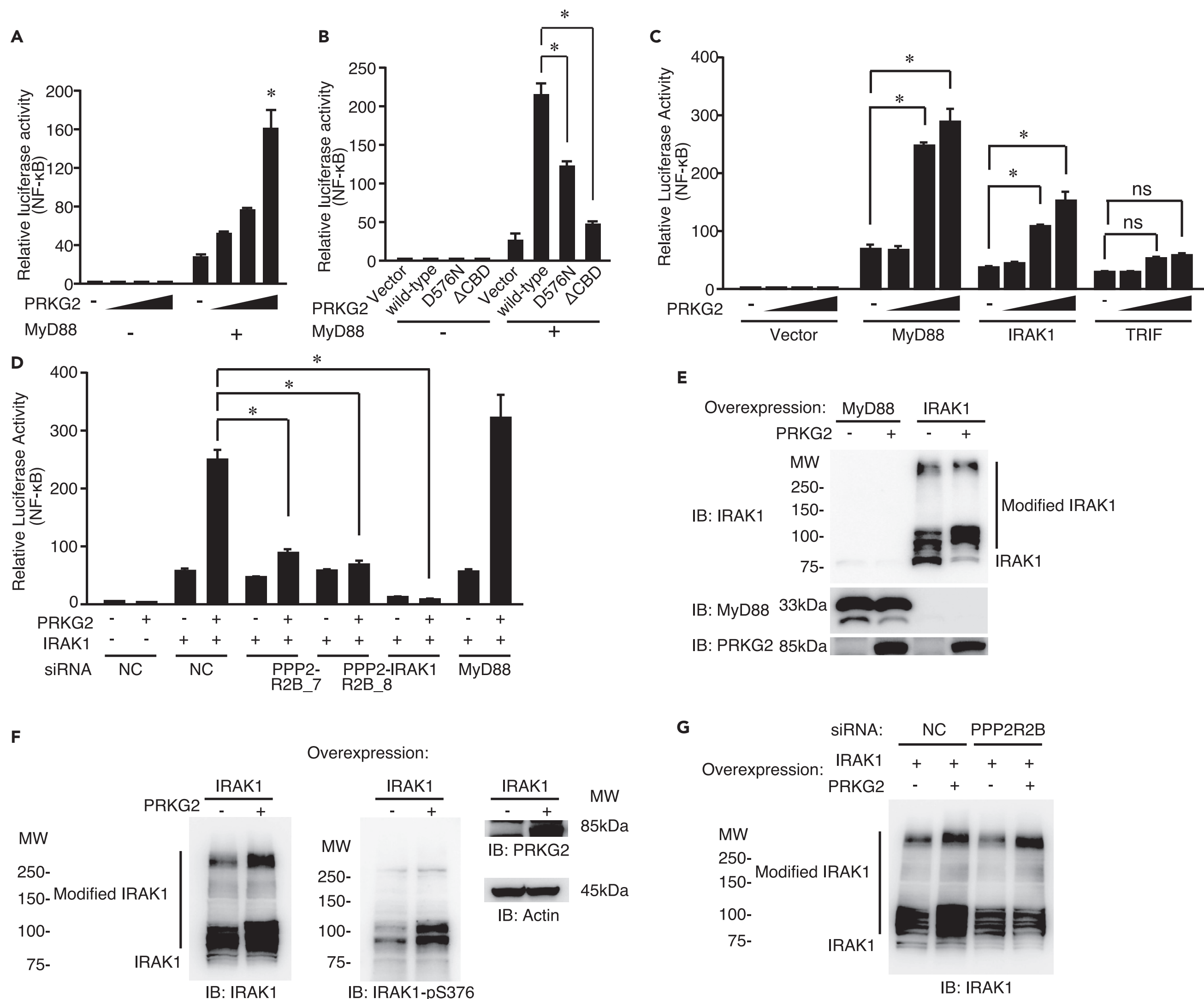

Figure 6. Membrane-bound cGMP-dependent protein kinase, PRKG2, and protein phosphatase 2A are involved in modulating NF-κB activation in human cells

(A) Membrane-bound cGK, PRKG2, enhances MyD88-dependent activation of NF-κB in human HEK293 cells.

(B) Effects of kinase-defective PRKG2 mutant, D576N, and cGMP binding-domain PRKG2 mutant, ΔCBD, on MyD88-dependent NF-κB activation.

(C) Effects of co-expression of PRKG2 on IRAK1-dependent NF-κB activation and on TRIF-dependent NF-κB activation in human HEK293 cells.

(D) Effects of siRNA-mediated targeting of human protein phosphatase 2A (PP2A) subunit, PPP2-R2B, IRAK1, and MyD88 on the enhanced IRAK1-dependent NF-κB activation by PRKG2. Two different siRNAs targeting PPP2-R2B (7 and 8) were used.

(E and F) Effects of co-expression of PRKG2 on the modification and phosphorylation (pS376, (F) of IRAK1 in human HEK293 cells.

(G) Effects of siRNA-mediated targeting of human PP2A subunit, PP2-R2B, on the enhanced modification of IRAK1 by PRKG2. $*P < 0.05$, ns $P > 0.1$, Student's t test.

Data are represented as mean, and error bars indicate standard deviation. Data shown are representative of at least three independent experiments.

homolog of Pelle (Daigneault et al., 2013). We found that IRAK1-dependent activation of NF-κB was enhanced by PRKG2 in human cells as well as in *Drosophila*, whereas TRIF (another adaptor protein for TLR signaling)-dependent NF-κB activation was not (Figure 6C). The enhanced IRAK1-dependent NF-κB activation by PRKG2 was suppressed by siRNA-mediated targeting IRAK1 and the PP2A regulatory subunit B beta (PPP2-R2B, Twins homolog) but not by targeting MyD88, indicating that PP2A is required to enhance the IRAK1-dependent activation of NF-κB by PRKG2, as in *Drosophila* (Figure 6D). The siRNA-mediated silencing of MyD88 reduced the enhanced MyD88-dependent NF-κB activation induced by PRKG2 (Figure S6G). Okadaic acid, a PP2A inhibitor, inhibited both the enhanced IRAK1-dependent and MyD88-dependent NF-κB activation by PRKG2 in HEK293 cells (Figure S6H), suggesting that the phosphatase

activity of PP2A is required for the cGMP-signaling-mediated modulation of NF-κB signaling. We also investigated IRAK1 modification (Figure 6E), as it undergoes phosphorylation and ubiquitination when activated (Tun-Kyi et al., 2011). PRKG2 expression increased modified IRAK1, including phosphorylation levels at serine 376, which is essential for IRAK1 activation (Figure 6F). In contrast, MyD88 expression was not affected by PRKG2 (Figure 6E). This enhanced modification of IRAK1 by PRKG2 was inhibited by siRNA-mediated targeting of PPP2-R2B (Figure 6G). These findings suggest that, in addition to conservation of the Toll and TLR pathways in innate immunity, modulation of NF-κB activation by a membrane-bound cGK and a protein phosphatase, PP2A, in the cGMP signaling pathway is also evolutionarily conserved in insects and mammals.

DISCUSSION

In the present study, we set out to address immune response modulation by cGMP signaling and receptor guanylate cyclases. We show that NF-κB (Toll pathway) signaling in the *Drosophila* immune response is regulated by the activation of both Toll-receptor-dependent signaling and Gyc76C-dependent cGMP signaling.

Our findings indicate the presence of a dual receptor system that allows for modulation of the intensity of the *Drosophila* immune response depending on the situation; activating both receptors induces a strong immune response. Further, Gyc76C is activated by ModSP and Psh, members of the serine protease cascade that cleaves the Spz precursor, suggesting that, as the Toll receptor ligand, the Gyc76C ligand is also produced endogenously by ModSP and Psh activation. A peptide, NPLP1-VQQ, is a reported ligand of Gyc76C in response to salt stress (Overend et al., 2012). NPLP1-VQQ, however, is not likely to be a ligand for Gyc76C in response to Gram-positive bacterial infections, because a null mutant of *Nplp1* encoding the NPLP1-VQQ precursor $Nplp^{EY11089}$ is not susceptible to Gram-positive bacterial infections, in contrast to the *gyc76C* mutant (data not shown). Additional studies are needed to identify and analyze the Gyc76C ligand and/or its precursor to elucidate how NF-κB signaling and *Drosophila* immunity are modulated by the dual receptor system we identified here.

The present study demonstrated that, in parallel with the Toll receptor, Gyc76C mediates ModSP-dependent and Psh-dependent NF-κB activation. ModSP and Psh are activated by both Gram-positive bacteria and fungi. $gyc76C^{KG03723}$ mutant adults and larvae, however, are resistant to fungal infection (Figures 3A, S4E, and S4F), in contrast to Gram-positive bacterial infections (Figures 3A and S4A). *modSP;psh* double mutants are susceptible to fungal infection under the same conditions (Figures S4E and S4F). Therefore, Psh- and ModSP-dependent NF-κB activation through the Toll receptor is sufficient for host defense against fungi. On the other hand, cGMP-pathway-mediated NF-κB modulation through Gyc76C has a crucial role in host defense against Gram-positive bacteria. The enhanced activation of NF-κB signaling by both receptors, the Toll receptor and Gyc76C, could be required for host defense against Gram-positive bacteria with more rapid proliferation than fungi. We recently reported that Gyc76C mediates hemocyte proliferation in a distinct manner from the cGMP pathway (Iwashita et al., 2020). A small GTPase, Ras85D, mediates Gyc76C-dependent hemocyte proliferation, and the loss-of-function of *Ras85D* causes host susceptibility against Gram-positive bacterial infection (Iwashita et al., 2020). Therefore, the Gyc76C-dependent cellular response may also be involved in host defense against Gram-positive bacteria.

Our results also demonstrate that NF-κB signaling activation is modulated by a membrane-bound cGK and PP2A in fly and human cells. cGMP levels are increased in *Drosophila* by the overexpression of ModSP as well as the overexpression of Gyc76C (Figures 1D and 2E), whereas no Gyc76C-dependent increase in cGMP is detected in *Drosophila* following Gram-positive bacterial infections (data not shown). Gyc76C is a membrane receptor that converts GTP to cGMP, and DG2 is a membrane-localized cGMP-dependent protein kinase. Moreover, DG2 physically associates with dMyd88 to form a signaling complex with Tube and Pelle after binding to the intracellular Toll/IL-1 receptor homologous domain of the activated Toll receptor. It is possible that cGMP is produced by Gyc76C at a specific membrane region under physiologic conditions, which could initiate the physical and functional interactions of DG2 with the dMyd88-dependent signaling complex. A higher cGMP level is not induced by bacterial infections, whereas it is induced by overexpression of Gyc76C and ModSP. We speculate that cGMP is rapidly degraded by PDEs *in vivo*, and therefore, no increase is observed in cGMP during infection. On the other hand, in the case of overexpression, cGMP is continuously produced, and its increase is detected. In mammals, activation of the dMyd88-dependent signaling complex induces IRAK1 phosphorylation and ubiquitination

(Tun-Kyi et al., 2011). Our study demonstrates that IRAK1 modifications, including phosphorylation at serine 376, which is essential for IRAK1 activation, are enhanced by the expression of PRKG2, a membrane-bound cGK, in human cells. The enhanced modification of IRAK1 is inhibited by PP2A knockdown, which inhibits the PRKG2-dependent enhancement of NF-κB activation. Therefore, the complex regulation of the phosphorylation and de-phosphorylation by two protein kinases, IRAK4 and IKAK1, in NF-κB signaling; a protein kinase, PRKG2; and a protein phosphatase, PP2A, in cGMP signaling may be involved in immune response modulation. Gyc76C is reported to be required for activation of the IMD pathway, another NF-κB pathway in *Drosophila*, in adult Malpighian tubules in response to salt stress (Overend et al., 2012). The present study demonstrated that Gyc76C activates the Toll pathway in larvae. Together these findings indicate that Gyc76C-mediated signaling modulates 2 NF-κB pathways, the IMD and Toll pathways, in a context-dependent manner in *Drosophila*. Further detailed studies are required to clarify how the cGMP signaling pathway modulates NF-κB signaling.

Our findings also suggest that the modulation of NF-κB activation by the cGMP signaling pathway in innate immune responses is evolutionarily conserved. For example, our findings show that a membrane-bound cGK, PRKG2, and PP2A are involved in modulating NF-κB activation in human cells, as in *Drosophila*. Other similarities between animals and plants in the regulation of immune responses include AtPepR1, an *Arabidopsis* receptor that bears an extracellular leucine-rich repeat, similar to that of the Toll receptor, and an intracellular guanylyl cyclase domain, similar to Gyc76C, with guanylate cyclase activity that produces cGMP (Qi et al., 2010). AtPepR1 is reported to be involved in the expression of pathogen-defense genes through cyclic-nucleotide-gated ion channel (Cng) 2, a cGMP-activated Ca^{2+} channel. The findings of Qi et al. (2010) together with those of the present study suggest that an extracellular leucine-rich repeat and intracellular guanylyl cyclase are involved in mediating innate immune responses in both animals and plants.

Limitations of study

This study demonstrated that NF-κB signaling in the *Drosophila* immune response is regulated by the activation of both Toll-receptor-dependent and Gyc76C-dependent cGMP signaling. Although we demonstrated that cGMP signaling is required for host defense against Gram-positive bacterial infections, the physiological conditions under which NF-κB signaling is modulated by cGMP signaling and the mechanisms by which it is controlled remain unclear. Future studies are needed to identify the ligands for Gyc76C and to clarify the physiological conditions under which Gyc76C is activated by the ligands.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

H.K, S.I., T.K., A.G., and N.F. performed many of the experiments in this study with input from S.K.; H.U. and R.W. performed the ModSP experiments; A.H., Y.M., and L.L.T. performed the larval infection experiments; H.I. performed the PRKG2 analyses; K.N. performed the gain-of-function screen and identified *CG3216*; T.T. performed the gain-of-function screen; M.N. and T.A. performed the GNBP-1 and Psh experiments; T.A. and H.U. performed the Gyc76C ligand assay; C.T. performed the *C. glabrata* infection experiments; S.Y. performed the okadaic acid experiments; H.S and M.F. performed the epistatic analyses; Y.O. promoted this study; T.A. provided GS lines for the gain-of-screen; S-A.D. and J.A.T.D. designed the cGMP studies; and S.K. provided overall coordination with respect to conception, design, and supervision of the study and wrote the manuscript with comments from co-authors. H.K., S.I., T.K., A.G., and N.F. contributed equally to the study. All authors discussed the results.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit IgG anti-IRAK1	Cell signaling	4359; RRID:AB_490853
Rabbit monoclonal anti-MyD88	Cell signaling	4283; RRID:AB_10547882
Mouse monoclonal anti-actin	Abcam	Ab3280; RRID:AB_303668
Rabbit IgG anti-PRKG2	Sigma	K0893; RRID:AB_1855711
Rabbit IgG anti-pS376-IRAK1	Pierce	Discontinued
Bacterial and virus strains		
E. coli (K-12)	Dr. Sekimizu	N/A
E. carotovora carotovora 15	Dr. Lemaitre	N/A
S. aureus	ATCC	10801
S. saprophyticus	GTC	0205
E. faecalis	IFO	12964
B. bassianna	ATCC	74040
C. glabrata	ATCC	2001
Chemicals, peptides, and recombinant proteins		
Trizol reagent	Thermo Fisher Scientific	15596018
ReverTra Ace	TOYOBO	TRT-101
Critical commercial assays		
Dual-glo Luciferase Assay System	Promega	E2920
Light Cycler FastStart DNA Master SYBR Green I	Roche	12239264001
Deposited data		
Microarray data	This paper	GSE108508, GSE108509, and GSE108510
Experimental models: Cell lines		
D. melanogaster:DL1 cells	*Drosophila* RNAi Screening Center	N/A
Human, HEK293 cells	ATCC	CRL-1573
Experimental models: Organisms/strains		
D. melanogaster: UAS-spz	Dr. Reichhart and Dr. Imler	Science **297,** 114-116 (2002).
D. melanogaster: UAS-dg2(P1A)	Dr. Davies	J. Biol. Chem. **279,** 40026-40034 (2004).
D. melanogaster: UAS-dg2(P2A)	Dr. Davies	J. Biol. Chem. **279,** 40026-40034 (2004).
D. melanogaster: UAS-dg2-RNAi	VDRC	38319
D. melanogaster: UAS-ModSP	Dr. Lemaitre	Proc. Natl. Acad. Sci. U.S.A. **106,** 12442–12447 (2009)
D. melanogaster: *psh*; *modSP*	Dr. Lemaitre	Proc. Natl. Acad. Sci. U.S.A. **106,** 12442–12447 (2009)
D. melanogaster: c564-GAL4	Dr. Perrimon	N/A
D. melanogaster: Cg-GAL4	Bloomington Stock Center	7011
D. melanogaster: Df(3L)Exel9061	Bloomington Stock Center	7946
D. melanogaster: spz^{rm7}	Dr. Anderson	Cell **76,** 677-688 (1994)
D. melanogaster: $Relish^{E20}$	Dr. Hultmark and Dr. Reichhart	Mol. Cell **4,** 827-837 (1999)

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
D. melanogaster: imd[1]	Dr. Reichhart	Dev. Cell **1**, 503-514 (2001)
D. melanogaster: dMyd88^{kra1}	Dr. Imler	Mech. Dev. **120**, 219-226 (2003)
D. melanogaster: UAS-Gyc76C	Dr. Kolodkin	J. Neurosci. **24**, 6639-6649 (2004)
D. melanogaster: Toll$^{1\text{-}RXA}$ and Toll^{r632}	Dr. Lemaitre	Nusslein-Volhard collection
D. melanogaster: DD1	Dr. Ferrandon	Biotechniques **30**, 594-598, 600-601 (2001)
D. melanogaster: Pelle^{2} and Pelle^{21}	Dr. Imler	Cell **86**, 973-983 (1996)
D. melanogaster: Dif/Dorsal^{J4}	Dr. Ip	Genes Dev. **13**, 792-797 (1999)
D. melanogaster: gyc76C^{KG03723}	Dr. Kolodkin	J. Neurosci. **24**, 6639-6649 (2004)
D. melanogaster: UAS-Gyc76C^{D945A}	Dr. Kolodkin	J Neurosci. **24**, 6639-6649 (2004)
D. melanogaster: UAS-PDE5/6	Dr. Davies	Biochem. J. **393(Pt 2)**, 481-488 (2006)
D. melanogaster: C729-GAL4	Bloomington Stock Center	6983
D. melanogaster: UAS-PGRP-LC	Dr. Royet	Nature **416**, 640-644 (2002)
D. melanogaster: UAS-Toll^{10B}	Dr. Reichhart	N/A
D. melanogaster: UAS-imd	Dr. Lemaitre	Dev. Cell **1**, 503-514 (2001)
D. melanogaster: NP2610-GAL4	National Institute of Genetics	112982
D. melanogaster: UAS-Psh	Dr. Reichhart	Science **297**,114-116 (2002)
D. melanogaster: UAS-GNBP-1	Dr. Lemaitre	Science **302**, 2126-2130 (2003)
Oligonucleotides		
Primers for CG3216, 5′-CCGGAATTCATGCATCTGCTTGGGATCAGCATC -3′ (F); 5′- TTGCGGCCGCTACTTTCCCATGCCGGCGA -3′ (R)	This paper	N/A
Primers for Rp49, 5′- AGATCGTGAAGAAGCGCACCAAG -3′ (F); 5′- CACCAGGAACTTCTTGAATCCGG -3′(R)	This paper	N/A
Primers for Gyc76C, 5′- AGCTACCCCAACTGGGAGAT -3′(F); 5′- TGACTCGAGTGCACTTCACC -3′ (R)	This paper	N/A
Primers for CG3216, 5′- AGTGGTCATCCTGTCGGTTC -3′ (F); 5′- TCATCCTTCATCTCCCAACC -3′ (R)	This paper	N/A
Primers for Gyc32E, 5′- AGAACGGAAGTCGTCATGCT -3′ (F); 5′- CCTCGCTTCAGAAGTTCAGC -3′ (R)	This paper	N/A
Primers for CG31183, 5′- CTGAAGCGCATGGAACTGTA -3′ (F); 5′- GGTGTGCAGGCCTATCCTTA -3′ (R)	This paper	N/A
Primers for Drs, 5′- TTGTTCGCCCTCTTCGCTGTCCT -3′ (F); 5′- GCATCCTTCGCACCAGCACTTCA -3′ (R)	This paper	N/A
Primers for Def, 5′- TTGAACCCCTTGGCAATGCA -3′ (F); 5′- AGTTCTTCGTTCTCGTGGCT -3′ (R)	This paper	N/A
Primers for Mtk, 5′- AACTTAATCTTGGAGCGA -3′ (F); 5′- CGGTCTTGGTTGGTTAG -3′ (R)	This paper	N/A
Primers for Dpt, 5′- GTTCACCATTGCCGTCGCCTTAC -3′ (F); CCCAAGTGCTGTCCATATCCTCC -3′ (R)	This paper	N/A
Primers for Att, 5′- GTGGTGGGTCAGGTTTTCGC -3′(F); TGTCCGTTGATGTGGGAGTA -3′(R)	This paper	N/A
Primers for CecA1, 5′- CATCTTCGTTTTCGTCGCTC -3′ (F); 5′- CGACATTGGCGGCTTGTTGA -3′ (R)	This paper	N/A
Primers for Droc, 5′- CCATCGTTTTCCTGCT -3′ (F); 5′- CTTGAGTCAGGTGATCC -3′(R)	This paper	N/A
Primers for dg2, 5′- ATTACTGGTCGCTGGGAGTG -3′ (F); 5′- AGAAGCCATCGAACCATTTG -3′ (R)	This paper	N/A

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Primers for PDE5/6, 5′- TTGCAATCAGCAATCTCAGC -3′ (F); 5′- CTGCGAAGGACTCGTAGGAC -3′ (R)	This paper	N/A
Primers for GFP-RNAi, 5′-TAATACGACTCACTATAGGGAGACCACGGAGG AGGACTTTCACTGGAG -3′ (F); 5′-TAATACGACTCACTATAGGGAGACCACGGAG GAGGACTTTCACTGGAG -3′ (R)	This paper	N/A
Primers for dMyd88 RNAi, 5′- TAATACGACTCA CTATAGGGAGACCACTACAGATGGGCCAACC GCTGCCC -3′ (F); 5′- TAATACGACTCACTATA GGGAGACCACTCCGAATGCTGGGAGTGGTCACC -3′ (R)	This paper	N/A
Primers for Tube RNAi, 5′- TAATACGACTCACTA TAGGGAGACCACAACTTCTGGTGCAGGCAG AGCTC -3′ (F); 5′- TAATACGACTCACTATAGGG AGACCACCGTGGCTGCAGCACTGGCTCCTG -3′ (R)	This paper	N/A
Primers for Pelle RNAi, 5′-TAATACGACTCACTATAGGGAGACCACCCTCAA TATTTGGGGCGGTCAG -3′ (F); 5′-TAATACGACTCACTATAGGGAGACCACGATGC TAAACCGCTGCTGCCAG -3′ (R)	This paper	N/A
Primers for Dif RNAi, 5′- TAATACGACTCACTA TAGGGAGACCACGGAACGCAATTTCGCTTC GCCTC -3′ (F); 5′- TAATACGACTCACTATAGG GAGACCACATTCCCAAGCTATTGAGTTCTGC -3′ (R)	This paper	N/A
Recombinant DNA		
human MyD88, ELAM-NF-κB luciferase reporter, and pRL-TK	Dr. Muta	N/A
dMyd88	Drosophila Genomic Resource Center	LD20892
cGKII	Origene	SC116254
IRAK1-HA	InvivoGen	pUNO2-hIRAK1-HA
TRIF	InvivoGen	pUNO2-hTRIF
Other		
Genome-wide RNAi Library	*Drosophila* RNAi Screening Center	DRSC 2.0

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Shoichiro Kurata (shoichiro.kurata.d5@tohoku.ac.jp).

Materials availability

This study did not generate new unique reagents.

Data and code availability

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Drosophila strains

In this study, we used UAS-spz (a gift from Dr. Reichhart and Dr. Imler), UAS-dg2(P1A) (a gift from Dr. Davies), UAS-dg2(P2A) (a gift from Dr. Davies), UAS-dg2-RNAi (VDRC, 38319), UAS-ModSP (a gift from Dr. Lemaitre), *psh*; *modSP* (a gift from Dr. Lemaitre), c564-GAL4 (a gift from Dr. Perrimon), Cg-GAL4 (Bloomington Stock Center, 7011), Df(3L)Exel9061 (Bloomington Stock Center, 7946), spz^{rm7} (a gift from Dr. Anderson), $Relish^{E20}$ (a gift from Dr. Hultmark and Dr. Reichhart), imd^{1} (a gift from Dr. Reichhart), $dMyd88^{kra1}$ (a gift from Dr. Imler), UAS-Gyc76C (a gift from Dr. Kolodkin), $Toll^{1\text{-}RXA}$(a gift from Dr. Lemaitre), $Toll^{r632}$ (a gift from Dr. Lemaitre), DD1 (a gift from Dr. Ferrandon), $Pelle^{2}$ (a gift from Dr. Imler), $Pelle^{21}$, (a gift from Dr. Imler) Dif/$Dorsal^{J4}$ (a gift from Dr. Ip), $gyc76C^{KG03723}$ (a gift from Dr. Kolodkin), UAS-$Gyc76C^{D945A}$ (a gift from Dr. Kolodkin), UAS-PDE5/6 (a gift from Dr. Davies), C729-GAL4 (Bloomington Stock Center, 6983), UAS-PGRP-LC (a gift from Dr. Royet), UAS-$Toll^{10B}$ (a gift from Dr. Reichhart), UAS-imd (a gift from Dr. Lemaitre), NP2610-GAL4 (National Institute of Genetics, 112982) , UAS-Psh (a gift from Dr. Reichhart), and UAS-GNBP-1 (a gift from Dr. Lemaitre). Flies were cultured in conventional cornmeal-agar food vials at 25°C unless otherwise noted. The conventional cornmeal-agar food contains 10.7 g cornmeal, 4.3 g yeast, 6.4 g glucose, 0.9 g agar, and 0.3 g ethyl 4-hydroxybenzoate in 100 mL. For survival experiments, males and females were mixed for each genotype.

Pathogens

The following pathogens were used for infection: *E. coli* (K-12), *E. carotovora carotovora 15*, *S. aureus* (ATCC10801, wood46), *S. saprophyticus* (GTC0205), *E. faecalis* (IFO12964), *B. bassianna* (ATCC74040), and *C. glabrata* (ATCC2001).

Cell lines

In this study, we used *Drosophila* DL1 cells (the *Drosophila* RNAi Screening Center) and human HEK 293 cells (ATCC).

METHOD DETAILS

Microbial infection

Flies were raised on a standard cornmeal-yeast agar medium. Flies (3-5 d after eclosion) were infected with bacterial strains by injecting ~70 nl of a suspension of each bacterial strain per fly. The optical density at 600 nm for each bacterial suspension was: *E. faecalis* (0.0001), *S. saprophyticus* (1.0), *S. aureus* (0.0001), *E. coli* (1.0), *Ecc15* (1.0), and *C. glabrata* (1.0). Survival experiments included 30 flies of each genotype at 28°C. Surviving flies were counted daily by transferring the flies to fresh vials (Takehana et al., 2004). For larval infection, overnight *S. aureus*, *E. coli*, *S. saprophyticus*, *or Ecc15* cultures were concentrated by centrifugation, the pellet was washed with phosphate-buffered saline, and the larvae were then pricked with a fine tungsten needle that had been dipped in a pellet of concentrated bacteria (Kenmoku et al., 2017).

cGMP enzyme-linked immunosorbent assay

Homogenates of 15 larvae were analyzed. cGMP determination was performed using a cGMP ELISA kit (Amersham) according to the manufacturer's instructions. Protein levels were quantified using the Bradford assay (Biorad) and protein levels were normalized.

DNA constructs

To generate *UAS-CG3216*, a PCR fragment was amplified with the following primers: forward 5′-CCGGAATTCATGCATCTGCTTGGGATCAGCATC -3′ and reverse 5′- TTGCGGCCGCTACTTTCCCA TGCCGGCGA -3′. The resulting fragment was subcloned into the *Eco*RI/*Not*I sites of a pUAST vector. In the case of *UAS-Gyc76C-RNAi*, the 333-bp PCR fragment (corresponding to nt 2395 – 2727 of GenBank accession number NM_079441) was subcloned into a pWIZ RNAi vector. To generate the *in vitro* expression vector under the control of a metallothionine promoter in *Drosophila* cells, full-length Gyc76C, dg2 P2, dMyd88, and Toll lacking residues 152-800 (designated TollΔLRR) were subcloned into a pMT vector (Invitrogen). For immunoprecipitation, dMyd88 with a V5 epitope tag (as a C-terminal fusion) and dg2 with a HA epitope tag (inserted between Gly15 and Gly16 in a putative linker region) were expressed. The cDNA clone of dMyd88 (LD20892) was obtained through the Drosophila Genomic Resource Center (Indiana University). The human MyD88 construct, ELAM-NF-κB luciferase reporter, and pRL-TK were provided by Dr. T.

Muta (Tohoku Univ.). The cDNA clone of cGKII, a human ortholog of dg2, was purchased from Origene (Cat. No. SC116254). Expression vectors for human IRAK1-HA and human TRIF were purchased from InvivoGen (pUNO2-hIRAK1-HA and pUNO2-hTRIF, respectively). All site-directed deletions, insertions, and point mutations in the constructs were engineered by PCR-mediated mutagenesis.

Cell culture, luciferase reporter assay, and RNAi

DL1 cells (provided by the *Drosophila* RNAi Screening Center) were maintained at 25°C in Schneider's Drosophila medium (Invitrogen) containing 10% (v/v) heat-inactivated fetal bovine serum. The scheme of the luciferase assay in *Drosophila* cells was described previously (Sun et al., 2002). To monitor the Toll pathway activity, 4.0×10^4 cells/well in a 96-well plate were transfected with 40 ng of pGL3-Drosomycin-Luciferase (Drs-Luc) reporter vector and 10 ng of an Actin5C-lacZ transfection control vector together with the indicated *in vitro* expression vectors using Effectene (Qiagen) following the manufacturer's recommendations. At 12 to 16 h after transfection, expression was induced by adding 500 μM CuSO4 and incubated at 28°C for 2 d. DL1 cells were then lysed in Glo Lysis Buffer (Promega). Luciferase activity was measured with the One-Glo Luciferase Assay System (Promega) in a luminometer (SpectraMax L-TYA, Molecular Devices). β-Galactosidase (β-gal) activity was measured with β-gal assay buffer (100 mM HEPES pH 7.3/150 mM NaCl/10 mM $MgCl_2$/0.5 mg/ml chlorophenol red-β-D-galactopyranoside; Roche), and the values of the absorbance of free chlorophenol red at 595 nm were used to normalize the variability in transfection efficiency. For RNAi and the dual-glo assay, DL1 cells (1.0×10^5) were plated in 96-well plates and transfected with 100 ng dsRNA, 40 ng pGL3-Drosomycin-Firefly luciferase reporter vector, and 10 ng pAc5.1-Renilla luciferase transfection control vector together with the indicated *in vitro* expression vectors using the Effectene transfection reagent (Qiagen). At 24 h after transfection, expression was induced by the addition of CuSO4 at a final concentration of ~1.0 mM and cells were incubated at 28°C for another 24 h. Luciferase activity was measured with the Dual-glo Luciferase Assay System (Promega) using the SpectraMax L-TYA luminometer (Molecular Devices). HEK293 cells were maintained at 37°C in 5% CO_2. To monitor NF-κB activation, 20,000 cells/well in 96-well plates were transfected with 20 ng of ELAM-NF-κB luciferase and 20 ng of pRL-TK together with the expression vectors using Lipofectamine LTX (Invitrogen). After 48 h of transfection, relative luciferase activity was measured using the Dual-Glo Luciferase Assay System (Promega).

Assay for Gyc76C ligand activity

Fifty larvae overexpressing *lacZ* or *ModSP* were homogenized with 500 μL of 0.1% (v/v) trifluoroacetic acid (Wako) in H_2O using a tissue homogenizer (Bertin Technologies, Precellys 24). The resulting homogenate was centrifuged for 10 min at 20,000g. The clear supernatant was evaporated in a Spin Driver (TAITEC, VC-96R) and solved in phosphate-buffered saline (PBS) as an acid extract. DL1 cells transfected with a *Drs*-Firefly luciferase reporter vector and an *Actin5C*-Renilla luciferase transfection control vector were incubated with the acid extract for 3 h. The luciferase reporter assay was carried out as described above. Heat treatment was performed at 100°C for 1 min. The ultrafiltration membrane with a molecular weight cut-off of 10,000 (Millipore, Amicon Ultra-4 10K) was used to estimate the molecular weight of the Gyc76 ligand. One arbitrary unit (AU) of the Gyc76C ligand activity was defined as the amount that showed 0.5 relative luciferase activity (firefly/renilla), calculated using a calibration curve created using the same assay.

Total RNA isolation and real-time PCR

Total RNAs were isolated from each genotype of ~20 flies with Trizol reagent (GIBCO/BRL). Total RNA (1 μg) was used for cDNA synthesis with ReverTraAce reverse transcriptase (Toyobo) and oligo(dT) 15 primer (Promega). Using the first-strand cDNA (0.5 μl), real-time PCR was performed using a LightCycler (Roche Diagnostics). *Rp49* was used as the internal control. The primers used for real-time PCR were as follows (F=forward, R=reverse):

Rp49: AGATCGTGAAGAAGCGCACCAAG (F); CACCAGGAACTTCTTGAATCCGG (R)

Gyc76C: AGCTACCCCAACTGGGAGAT (F); TGACTCGAGTGCACTTCACC (R)

CG3216: AGTGGTCATCCTGTCGGTTC (F); TCATCCTTCATCTCCCAACC (R)

Gyc32E: AGAACGGAAGTCGTCATGCT (F); CCTCGCTTCAGAAGTTCAGC (R)

CG31183: CTGAAGCGCATGGAACTGTA (F); GGTGTGCAGGCCTATCCTTA (R)

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Drs: TTGTTCGCCCTCTTCGCTGTCCT (F); GCATCCTTCGCACCAGCACTTCA (R)

Def: TTGAACCCCTTGGCAATGCA (F); AGTTCTTCGTTCTCGTGGCT (R)

Mtk: AACTTAATCTTGGAGCGA (F); CGGTCTTGGTTGGTTAG (R)

Dpt: GTTCACCATTGCCGTCGCCTTAC (F); CCCAAGTGCTGTCCATATCCTCC (R)

Att: GTGGTGGGTCAGGTTTTCGC (F); TGTCCGTTGATGTGGGAGTA (R)

CecA1: CATCTTCGTTTTCGTCGCTC (F); CGACATTGGCGGCTTGTTGA (R)

Droc: CCATCGTTTTCCTGCT (F); CTTGAGTCAGGTGATCC (R)

dg2: ATTACTGGTCGCTGGGAGTG (F); AGAAGCCATCGAACCATTTG (R)

PDE5/6: TTGCAATCAGCAATCTCAGC (F); CTGCGAAGGACTCGTAGGAC (R)

Colony forming unit assay

Flies were collected at 0, 6, 24, and 48 h after injecting each bacterial strain and sterilized with 70% ethanol. At total of 10 flies of each genotype was homogenized in 500 μl of the appropriate bacterial medium, serially diluted, and plated onto the appropriate plates (Luria Bertani medium for *E. coli*, *Ecc15*, and *E. faecalis*; nutrient broth medium for *S. aureus* and *S. saprophyticus*).

Microarray analysis

Total RNA from *Drosophila* adult flies homogenized in TRIzol (Invitrogen) was isolated using an RNeasy kit (Qiagen). The RNA quality was checked using an Agilent Bioanalyzer 2100 (Agilent Technologies). Total RNA (1 μg) was amplified and labeled as complementary RNA using an IVT Labeling Kit (Affymetrix). Affymetrix *Drosophila* Genome 2.0 arrays were hybridized with 30 μg labeled complementary RNA, washed, stained, and scanned. Changes in expression are shown as the signal log ratio. The most widely used alternative transformation of the ratio is the logarithm base 2, which has the advantage of producing a continuous spectrum of values and treating upregulated and downregulated genes similarly (Quackenbush, 2002). The accession numbers for the microarray data are GEO: GSE108508, GSE108509, and GSE108510. Raw data of all processed microarray analyses are available at the following *Drosophila* microarray database at the Tohoku University web site: http://www.pharm.tohoku.ac.jp/~seimei/m_array/index.htm.

Genome-wide RNAi screening and analysis

We used the DRSC Genome-wide RNAi Library (DRSC 2.0), 66 plates of 384-well format microplates with dsRNAs covering ~13,900 genes, for genome-wide RNAi screening. Drosophila DL1 cells were transfected with vectors and dsRNA using Effectene Transfection reagent (QIAGEN) according to the procedures described below. In each well of the 384-well plate, 10 ng pGL3-Drosomycin-Firefly luciferase reporter vector, 10 ng pAc5.1-Renilla luciferase transfection control vector, 10 ng pMT-dMyd88-v5his, 20 ng pMT-Gyc76C, 20 ng pMT-DG2P2, 0.4 μL Enhancer, and 0.5 μL Effectene were diluted in 9.3 μL buffer EC, and the solution (~10 μL) was immediately mixed with 80 ng dsRNA in each well of the screening plate. After 10-min incubation, 35 μL of DL1 cells (1.0 x 10^5 cells/mL) was transfected with the lipid-nucleotide complex in the well and cultured at 28°C for 24 h. Overexpression of dMyd88 was induced by adding CuSO4 at a final concentration of 0.5 mM for 48 h. Firefly-luciferase activity (Drosomycin-promoter activity) and Renilla-luciferase activity (cell viability and transfection efficiency) were measured using a SpectraMax L-TYA luminometer (Molecular Devices) with the Dual-glo luciferase assay system (Promega). Data analysis was performed according to the median + k median-absolute deviation method described previously (Birmingham et al., 2009). Relative luciferase activities (RLA = Firefly/Renilla) were calculated and transformed as a percentage of the median RLA in the plate. For the first hits, wells under 40% of the median RLA were considered positive. We then compared the candidate genes between dMyd88 single-expression screening (Kanoh et al., 2015) and dMyd88/Gyc76C/DG2P2 co-expression screening. Finally, we obtained 29 specific hits by subtracting the candidate genes of dMyd88 single-expression screening from those of dMyd88/Gyc76C/DG2P2 co-expression screening.

QUANTIFICATION AND STATISTICAL ANALYSIS

The data are represented as mean with standard deviation. Statistical analyses between 2 groups were performed using the independent Student's *t*-test. Log-rank tests were performed to estimate the significance of differences in overall survival assays. $P<0.05$ was considered to indicate statistical significance.