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Ecdysone triggered *PGRP-LC* expression controls *Drosophila* innate immunity

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Throughout the animal kingdom, steroid hormones have been implicated in the defense against microbial infection, but how these systemic signals control immunity is unclear. Here, we show that the steroid hormone ecdysone controls the expression of the pattern recognition receptor *PGRP-LC* in *Drosophila,* thereby tightly regulating innate immune recognition and defense against bacterial infection. We identify a group of steroid-regulated transcription factors as well as two GATA transcription factors that act as repressors and activators of the immune response and are required for the proper hormonal control of PGRP-LC expression. Together, our results demonstrate that *Drosophila* use complex mechanisms to modulate innate immune responses, and identify a transcriptional hierarchy that integrates steroid signalling and immunity in animals.

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Introduction

Hormones are important regulators of many physiological processes including metabolism, development, reproduction as well as immune responses. In the context of immunity, pharmacologic application of glucocorticoids has multiple potent anti-inflammatory effects on immune cells (Necela and Cidlowski, 2004; Sternberg, 2006). In addition to the well-known immunosuppressive effects of glucocorticoids, many studies have revealed that physiological levels of glucocorticoids actually enhance the immune and inflammatory response (Galon *et al*, 2002; Shuto *et al*, 2002; Goulding, 2004; Hermoso *et al*, 2004; Sakai *et al*, 2004; Dhabhar, 2009; Busillo *et al*, 2011). Several other nuclear hormone receptors, including estrogen receptors (ERs), peroxisome proliferator-activated receptors (PPARs), vitamin D receptors (VDRs), retinoid-related orphan receptors (RORs), retinoid X receptors (RXRs) and liver X receptors (LXRs), have also been found to positively regulate innate immunity and proinflammatory cytokine expression (Tontonoz *et al*, 1998; Hong and Tontonoz, 2008; Jetten, 2009; Baeke *et al*, 2010; Nunez *et al*, 2010). All together, these reports exemplify the complex and sometimes contradictory regulation of innate immune and inflammatory responses by steroid/retinoid hormones and their receptors; therefore a more simple and genetically tractable system for studying the interface between steroids and innate immunity would be highly valuable.

Several studies have suggested that the steroid hormone 20-hydroxyecdysone (20E), a central regulator of development, metamorphosis and reproduction in insects (Riddiford, 1993; Kozlova and Thummel, 2000), also positively regulates the innate immune response in the fruit fly, *Drosophila melanogaster* (Meister and Richards, 1996; Dimarcq *et al*, 1997; Lanot *et al*, 2001; Sorrentino *et al*, 2002; Flatt *et al*, 2008; Stofanko *et al*, 2008; Zhang and Palli, 2009). In particular, 20E enhances the pathogen-induced expression of antimicrobial peptide (*AMP*) genes in both animals and cultured cell lines. These studies suggest that 20E affects AMP expression by regulating the immune deficiency (IMD) pathway, one of two NF-κB signalling pathways in *Drosophila,* which control the induction of *AMP* genes in response to DAP-type peptidoglycan (PGN) from the cell wall of Gram-negative and certain Gram-positive bacteria (Kaneko *et al*, 2006). However, the mechanisms whereby 20E modulates the IMD pathway to affect *AMP* gene induction remain unclear. In contrast, the mechanisms whereby 20E controls developmental events are well understood.

During development, 20E regulates gene expression through binding to a nuclear hormone receptor heterodimer consisting of the ecdysone receptor (EcR) and ultraspiracle (USP) proteins—orthologs of the vertebrate LXR and RXR receptors, respectively (King-Jones and Thummel, 2005). Ligand binding to the EcR/USP-receptor complex triggers the transcription of 'early' response genes, which themselves encode several different transcription factors (*e.g.,* the helix-turn-helix factor Eip93F, the zinc finger factor BR-C, the ETS domain factor Eip74EF and the nuclear hormone receptor Eip75B (Baehrecke and Thummel, 1995; Thummel, 1996; Mugat *et al*, 2000). Several other genes act as delayed early genes, including the nuclear hormone receptors *Eip78C* and *Hr46* (*DHR3)*, that further contribute to the 20E-triggered transcriptional hierarchy (King-Jones and Thummel, 2005). Here, we identify two novel and distinct mechanisms whereby this ecdysone-inducible transcriptional network controls the IMD immune response in *Drosophila*. Pattern recognition receptor (*PGRP-LC)* is a key sensor of DAP-type PGN, and the expression of this receptor, subsequent activation of IMD

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signal transduction and induction *AMP* gene expression, is critically dependent on five early ecdysone-inducible transcription factors (BR-C, Eip93F, Eip74EF, Eip78C and HR46) as well as two dGATA factors (Serpent (SRP), and Pannier (PNR)). However, these regulatory connections do not fully explain the 20E control of AMP induction; a second 20E-mediated mechanism, which robustly affects only a subset of *AMP* genes, is uniquely controlled by BR-C, SRP and PNR. Together, these results demonstrate that 20E functions through multiple, complex and partially overlapping transcriptional circuits to regulate the adult immune response in *Drosophila*.

Results

20E regulates the expression of PGRP-LC

Previously, we and others have demonstrated that the 20E modulates the induction of *AMP* genes in immune-challenged *Drosophila* cell culture or whole animals (Meister and Richards, 1996; Dimarcq *et al*, 1997; Flatt *et al*, 2008; Zhang and Palli, 2009). In particular, PGN-induced *AMP* gene induction is nearly undetectable without ~24 h pretreatment with 20E in S2* cells (Flatt *et al*, 2008). To identify potential downstream targets of 20E involved in modulating the immune response, microarrays were used to compare the transcriptomes of S2* cells exposed or not to 20E for 24 h. Analysis of the gene expression profiles of all known IMD pathway components identified several signalling factors, such as *bendless*, *effete*, *Diap2*, *Tab2*, *Relish*, *Caspar* and *kayak* that were modestly but significantly increased upon hormone treatment (Figure 1A), perhaps contributing to the enhancement of PGN-induced *AMP* gene expression. More striking, *PGRP-LC* gene expression was undetectable prior to 20E treatment but robustly induced by hormone treatment ($P = 0.007$). PGRP-LC is a critical bacterial sensing receptor required for activating IMD signalling following many bacterial infections (Choe *et al*, 2002; Gottar *et al*, 2002; Ramet *et al*, 2002; Leulier *et al*, 2003; Werner *et al*, 2003; Kaneko *et al*, 2004; Takehana *et al*, 2004; Kaneko *et al*, 2006). Validating the array data, qRT–PCR analysis of *PGRP-LC* expression showed tight hormonal control in S2* cells, with receptor expression preceding the inducibility of the *AMP* gene *Diptericin* (*Dpt*) by approximately 6 h (Figure 1B).

To begin to compare our cell-based results with the intact animal, we examined the modENCODE RNAseq data for the developmental profile of *PGRP-LC* expression (Graveley *et al*, 2010). *PGRP-LC* expression is roughly coincident with the developmental pulses of 20E (Riddiford, 1993; Dubrovsky, 2004; Warren *et al*, 2006) (Supplementary Figure S1). These observations suggest that 20E plays a major role in controlling *PGRP-LC* expression, both *in vivo* and in cell culture.

Ectopic PGRP-LC expression bypasses hormonal control by 20E

To investigate if regulation of *PGRP-LC* is the primary mechanism by which 20E controls the IMD pathway, a stable cell line expressing C-terminally FLAG-tagged PGRP-LCx from the copper-inducible metallothionein (MT) promoter was established. Fortuitously, we found expression of PGRP-LCx-FLAG to be 'leaky' in this cell line, with low-level expression occurring even in the absence of copper (Supplementary Figures S2 and S3, upper panels). As expected, 3 h of treatment with 100 μM $CuSO_4$ further increased the level of PGRP-LCx-FLAG (Supplementary Figures S2 and S3, lower panels). Surprisingly, these data (as well as our unpublished data with other metallothionein promoter-controlled transgenes) show that 20E downregulates the metallothionein promoter (Supplementary Figures S2 and S3); however, this phenomenon is independent of the 20E-control of *PGRP-LC* expression at its natural locus.

Next, IMD signalling events were evaluated in this PGRP-LCx-FLAG expressing cell line. Previous studies have shown that PGN-stimulation triggers the rapid cleavage and ubiquitination of the IMD protein, while the NF-κB precursor Relish is cleaved, phosphorylated and translocated to the nucleus (Stöven *et al*, 2000, 2003; Ertürk-Hasdemir *et al*, 2009; Paquette *et al*, 2010). Immunoblot analysis of lysates derived from the parental S2* cells showed that 20E pretreatment is required for PGN-induced IMD cleavage and ubiquitination as well as Relish cleavage and phosphorylation (Figure 2, left panels). On the other hand, in the PGRP-LCx expressing cells all of these PGN-triggered events occur upon PGN-stimulation independent of 20E pretreatment (Figure 2, right panels). To examine the nuclear translocation of Relish, the subcellular localization of YFP-tagged Relish was examined by confocal microscopy. In the parental S2* cells, translocation of Relish occurred only when PGN stimulation was preceded by 20E treatment (Supplementary Figure S4A and C), while in the PGRP-LCx-FLAG expressing cells nuclear translation was independent of 20E treatment (Supplementary Figure S4B and D). Together, these biochemical and microscopy analyses demonstrate that ectopic expression of the PGRP-LC receptor is sufficient to support PGN-induced IMD signal transduction, bypassing the need for the steroid hormone 20E.

Dual mechanisms of 20E regulation of AMP gene expression

To investigate whether the 20E-independent expression of the PGRP-LC receptor is sufficient to support PGN-induced transcription, *AMP* gene induction was quantified. In the parental cell line, PGN-triggered *AMP* gene expression was dependent on pretreatment with 20E (Figure 3, grey bars). Consistent with the biochemical analysis of IMD signalling (shown in Figure 2), the PGN-triggered expression of several *AMP* genes (*Cecropin A1* (*CecA1*), *Attacin A* (*AttA*), and *Defensin* (*Def*)) was readily observed without 20E pretreatment in the PGRP-LCx-FLAG expressing cell line (Figure 3, right panels). In fact, ectopic expression of PGRP-LCx in these cells led to modest expression of these *AMP* genes in the absence of any immune stimulus. On the other hand, the induction of other *AMP* genes (*Diptericin (Dpt), Metchnikowin (Mtk), Drosomycin (Drs)*) was still largely dependent on 20E pretreatment, even in the PGRP-LCx expressing cells (Figure 3, left panels, black bars).

Remarkably, these findings suggest two distinct mechanisms for 20E-control of *AMP* gene induction. The first mechanism involves the 20E-regulated expression of PGRP-LCx. Bypassing this control is sufficient to support PGN-mediated activation of IMD signal transduction and robust induction of a subset of *AMP* genes (*i.e., CecA1, AttA* and *Def*). A second hormone-control mechanism is absolutely required for the immune-induced expression of a distinct subset of *AMP* genes (*Dpt*, *Mtk* and *Drs*). This second mechanism appears

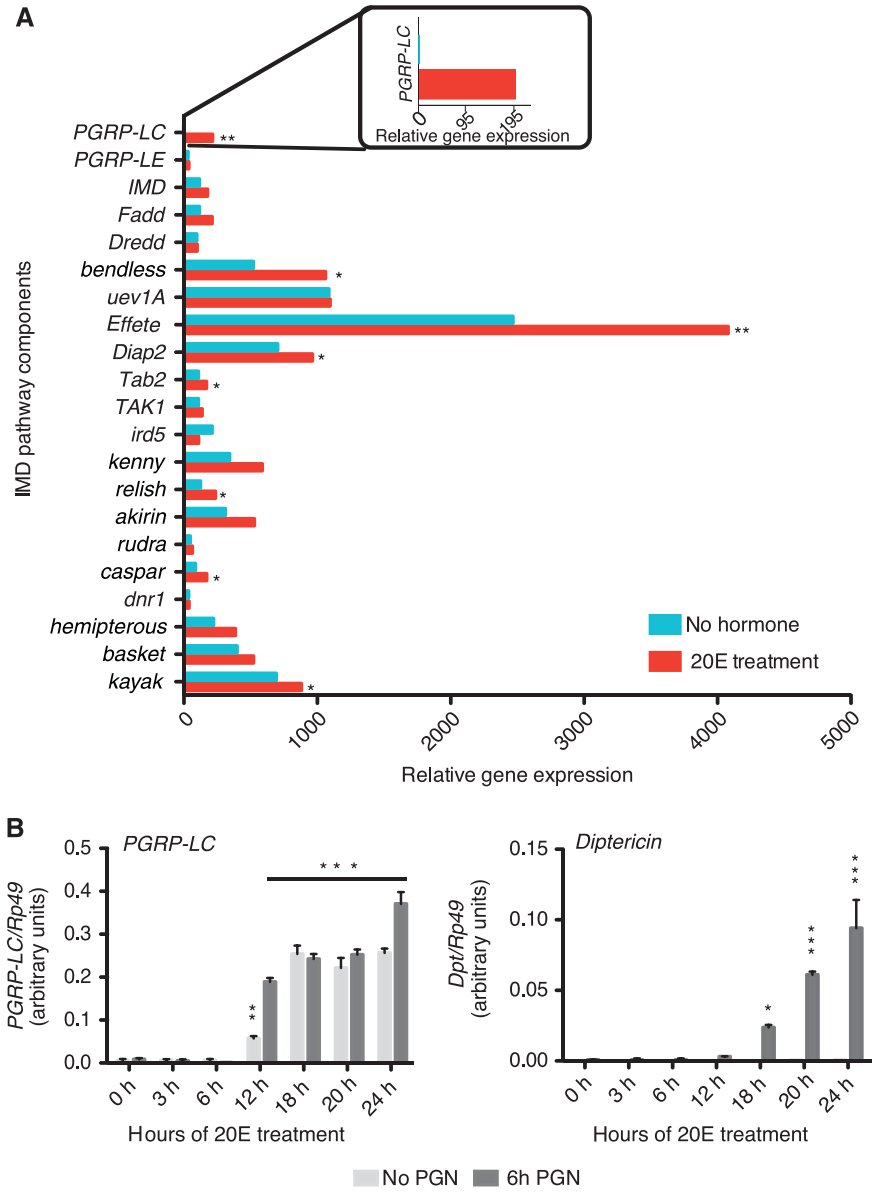

Figure 1 20E controls PGRP-LC expression. (**A**) Microarray expression profiles for IMD pathway components. Profiles were generated from triplicate samples of S2* cells before and after 24 h of treatment with 20E. The asterisks represent statistical significance (*P value <0.05; **$P<0.01$) calculated by unpaired t-test. (**B**) Real-time qRT–PCR analysis of *PGRP-LC* and *Dpt* transcripts from S2* cells that were exposed to 20E for various times, as indicated, and treated with PGN for 6 h or left untreated prior to harvest for RNA isolation. The mean of three independent biological replicates is shown, and error bars represent standard deviation. *PGRP-LC* levels at 12 h of hormonal treatment with or without PGN stimulation, are significantly increased compared with the untreated samples (0h), while *Diptericin* levels are significantly increased beginning at 18 h after hormonal treatment, only after PGN stimulation. *$P<0.05$, **$P<0.01$, ***$P<0.001$, as determined by one-way ANOVA with Tukey's multiple comparison test.

to function downstream of the cleavage and nuclear localization of Relish.

Classic 20E targets are required for the steroid control of IMD signalling

Ecdysone is known to directly regulate a battery of genes through the EcR and ecdysone-response elements found near target genes. Many direct targets of EcR are themselves transcription factors, which initiate a cascade of transcriptional programs downstream of this hormone (Thummel, 2002). Given the 12–18 h required to observe the effect of 20E on *PGRP-LC* expression and IMD signalling, it seems likely that secondary or tertiary targets of 20E/EcR signalling mediate the IMD-potentiating activity. Therefore, twelve 20E-inducible transcription factors, identified from the literature and our microarray data (Kozlova and Thummel, 2000; Thummel, 2002; King-Jones and Thummel, 2005), were analyzed for their role in 20E-mediated regulation of the IMD immune response (Supplementary Figure S5A and B). RNAi-mediated depletion of seven transcription factors, *br-c*, *Eip78C*, *Eip93F*, *Eip74EF*, *Hr46*, *srp* and *pnr*, reproducibly blocked 20E-supported PGN-induced *Dpt* and *CecA1* expression in S2* cells (Figures 4A and B). As controls, S2* cells were treated with *EcR* RNAi, which also prevented 20E immune potentiation (Flatt *et al*, 2008), or mock RNAi treatment (Figures 4A and B). In contrast, RNAi-mediated

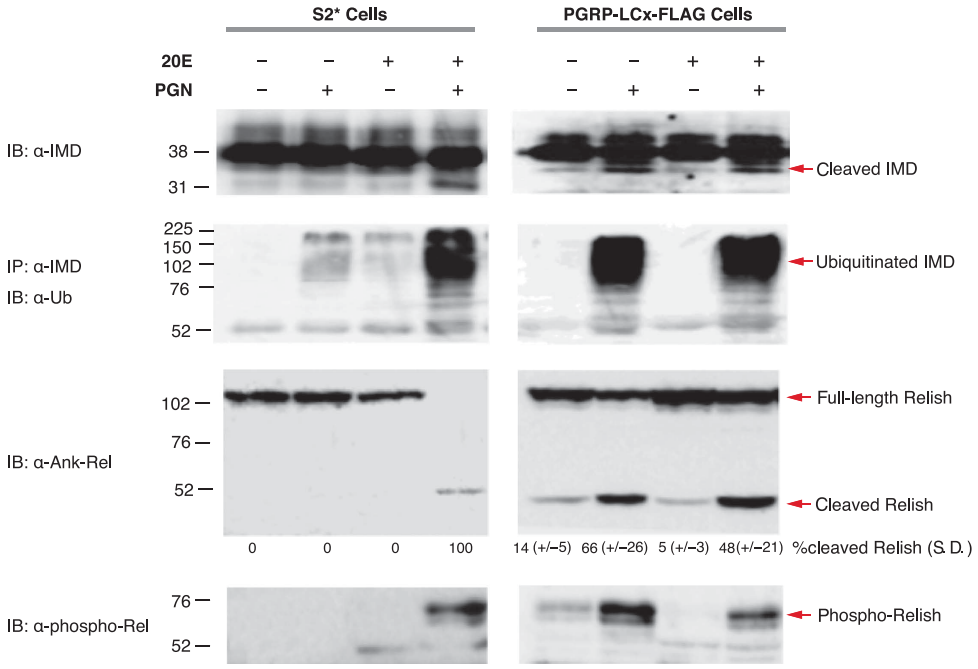

Figure 2 20E-independent IMD signalling in PGRP-LCx-FLAG expressing cells. Analysis of whole-cell lysates from parental S2* or PGRP-LCx-FLAG cells with or without 24 h pretreatment with 20E, followed by 10 min stimulation with PGN, as indicated. IMD cleavage was analyzed by immublotting (IB) while IMD ubiquitination was monitored by immunoprecipitation (IP)-IB (upper two panels); Relish cleavage and phosphorylation were analyzed by IB (lower two panels). The percent Relish cleavage was quantified by measuring the intensity of the relevant bands from three different experiments and the mean from three experiments (with s.d.) are shown.

depletion of *Eip75B* robustly enhanced 20E-supported PGN-induced *Dpt* and *CecA1* expression (Figures 4A and B), consistent with an earlier report that Eip75B is a negative regulator of IMD signalling (Kleino *et al*, 2005). The other four transcription factors analyzed, *ERR* (estrogen-related receptor), *Hsf* (heat shock factor), *Hnf4* (hepatocyte nuclear factor 4) and *luna* had no effect on 20E-supported PGN-induced *Dpt* expression (Supplementary Figure S6).

Since these results demonstrate that BR-C, Eip78C, Eip93F, Eip74EF, Hr46, SRP and PNR all play a role in 20E-mediated IMD signalling, the role of these transcription factors in the control of PGRP-LC was also quantified. *PGRP-LC* expression was signficantly reduced in S2* cells when *br-c*, *Eip93F*, *Eip78C*, *Eip74EF*, *srp*, *pnr*, *Hr46* or *EcR* were depleted by RNAi (Figures 4C, $***P<0.001$). Depletion of *Eip75B*, by contrast, increased *PGRP-LC* levels ($***P<0.001$), consistent with the higher levels of *Dpt* and *CecA1* expression observed with knockdown of this gene.

A similar RNAi analysis of these transcription factors was also performed in the cell line engineered to express PGRP-LCx-FLAG. Depletion of *br-c, srp, pnr* or *EcR* nearly abolished 20E-supported induction of *Dpt, Drs* and *Mtk,* even in the presence of PGRP-LCx ($***P<0.001$, $**P<0.01$), while targeting the other ecdysone-inducible factors had more modest and variable effects (Figures 5B, D, F). As expected, the expression of *AMP* genes was reduced in the parental S2* cells when any of these transcription factors were depleted by RNAi (Figures 5A, B, C). Together, these data argue that 20E signalling regulates IMD signalling through at least two distinct regulatory mechanisms. First, *PGRP-LC* expression is controlled by 20E through EcR and Eip78C, Eip93F, Eip74EF, HR46, BR-C, SRP and PNR. Second, the induction of a subset of *AMP* genes is additionally modulated by hormone, independent of Relish activation, through the activities of EcR, BR-C, SRP and PNR.

Ecdysone signalling is required for immune responses in adult flies

To examine the role of the 20E-signalling components in the immune response *in vivo*, dominant acting mutants affecting ecdysone signalling were exploited (Yoshihara and Ito, 2000; Ishimoto and Kitamoto, 2010). EcR^{NP5219}, which carries P-insertion in an *EcR* intron, and EcR^{A483T}, which carries a point mutation in the ligand-binding domain of EcR, both exhibit significantly decreased *Dpt* expression compared to their wild-type controls (*cn bw* or $w^{1118iso5}$, respectively) (Supplementary Figure S7A–D). Next, we examined the mutant *dominant temperature sensitive 3* (*DTS-3*), which is deficient for the production of 20E. At the fully restrictive temperature (29°C), *DTS-3* mutant displays dominant lethality during development due to a low ecdysone titer (Walker *et al*, 1987). However, *DTS-3/+* females can develop into adults if reared at 25°C, and have a 50% lower 20E titer than wild-type animals (Walker *et al*, 1987; Ishimoto *et al*, 2009). Similar to the *EcR* mutants, *DTS-3/+* mutant females reared at 25°C had significantly reduced *Dpt* expression following *E. coli* infection as compared to the wild-type control *Samarkand* ($P=0.0001$) (Supplementary Figure S7E), while the same strain reared at the permissive temperature (18°C) does not exhibit a significant decrease in *Dpt* induction (Supplementary Figure S7F) ($P=0.1$).

To further investigate the role of the transcription factors functioning downstream of 20E and EcR, an RNAi approach was used. By expressing transgenic hairpin-RNAs with Gal4/UAS system (Brand and Perrimon, 1993; Dietzl *et al*, 2007), *EcR*, *br-c*, *Eip78C*, *Eip93F*, *Eip74EF*, *Eip75B*, *Hr46*, *srp* or *pnr*

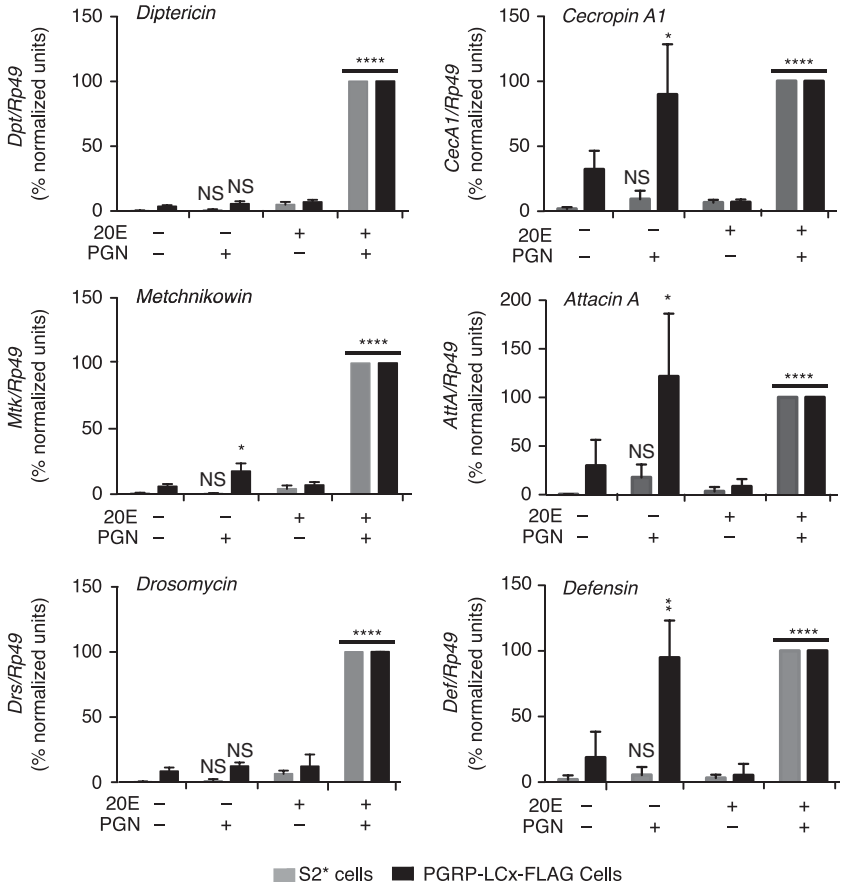

Figure 3 20E-dependent and -independent expression of *AMP* genes in PGRP-LCx-FLAG cells. qRT–PCR analysis of *Dpt, Mtk, Drs, CecA1, AttA* and *Def* expression from parental S2* (grey bars) and PGRP-LCx-FLAG cells (black bars). Cells, with or without 24 h of 20E pretreatment, were stimulated with PGN for 6 h (or not), and then harvested for RNA isolation, as indicated. *AMP* gene expression was normalized to *Rp49* levels, and then normalized between biological replicates and represented as a percentage of the expression level relative to the 20E treated and PGN-stimulated samples from each cell type. For each treatment, the values shown represent the mean of three independent experiments. Error bars represent standard deviations and $*P<0.05$, $**P<0.01$, $****P<0.0001$ were calculated using unpaired *t*-test for comparing the corresponding samples with or without hormonal treatment.

were depleted in the adult female fat body, the major site of systemic infection-induced *AMP* gene expression. Importantly, the *Yp1-GAL4* driver used for these studies does not activate gene expression until ~3 days after eclosion and only in females (Georgel *et al*, 2001). In contrast to the mutants of *EcR* and *DTS-3* used above, which interfere with ecdysone signalling throughout development, this system allows 20E signalling to be inhibited specifically during adulthood and only in the major immune responsive organ. Experimental *Yp1-GAL4>*UAS-RNAi animals and controls (*Yp1-GAL4* females alone and males with identical genotypes) were infected with *E. coli* and analyzed for *AMP* gene expression 24 h later. Similar to the cell-based studies, depletion of *EcR*, *br-c*, *Eip78C*, *Eip93F*, *Eip74EF*, *Hr46*, *srp* and *pnr* resulted in significantly reduced induction of *Dpt* and *CecA1* as well as reduced expression of *PGRP-LC* (Figures 6A–C). Note that males of the identical genotype showed normal AMP and PGRP-LC expression (Supplementary Figure S8A–C). Furthermore, *Eip75B* RNAi females showed significantly increased levels of the *AMP* genes as well as the receptor *PGRP-LC* (Figures 6A–C), thus confirming the role of Eip75B as a negative regulator of IMD signalling *in vivo*. These results demonstrate that 20E signalling can regulate IMD signalling through *br-c, Eip78C, Eip93F, Eip74EF, Hr46*, *srp* and *pnr* in adults, independent of the developmental properties of 20E.

Fly lines carrying the same *UAS-RNAi* constructs were also crossed with the *C564-GAL4* driver, which expresses GAL4 in the fat body, hemocytes, as well as some male reproductive tissues (Hrdlicka *et al*, 2002; Buchon *et al*, 2009). C564-Gal4->UAS-*EcR* RNAi or UAS-*br-C* RNAi were not viable to adulthood. However, *Dpt* expression levels in response to *E. coli* infection was significantly reduced, compared to control *C564-GAL4* driver alone animals, in all other RNAi lines (except *Eip75B* RNAi), in both males and females (Supplementary Figure S9 A-B). On the other hand, *C564-GAL4*-driven *UAS- Eip75B* RNAi significantly enhanced *Dpt* expression in females, consistent with its role as a negative regulator of IMD signalling. Together, these data demonstrate that ecdysone-signalling pathway has a critical role in regulating IMD signalling and PGRP-LC expression in adult flies.

To determine whether the reduction of infection-induced *AMP* gene expression observed with depletion of *EcR, br-c, Eip78C, Eip93F, Eip74EF*, *srp* or *pnr* also causes a functional decrease of the realized immune defense, flies were infected with the Gram-negative pathogen *Erwinia carotovora carotovora 15 (Ecc15)* and their survival monitored. RNAi expressing fly strains were rapidly killed by this infection as

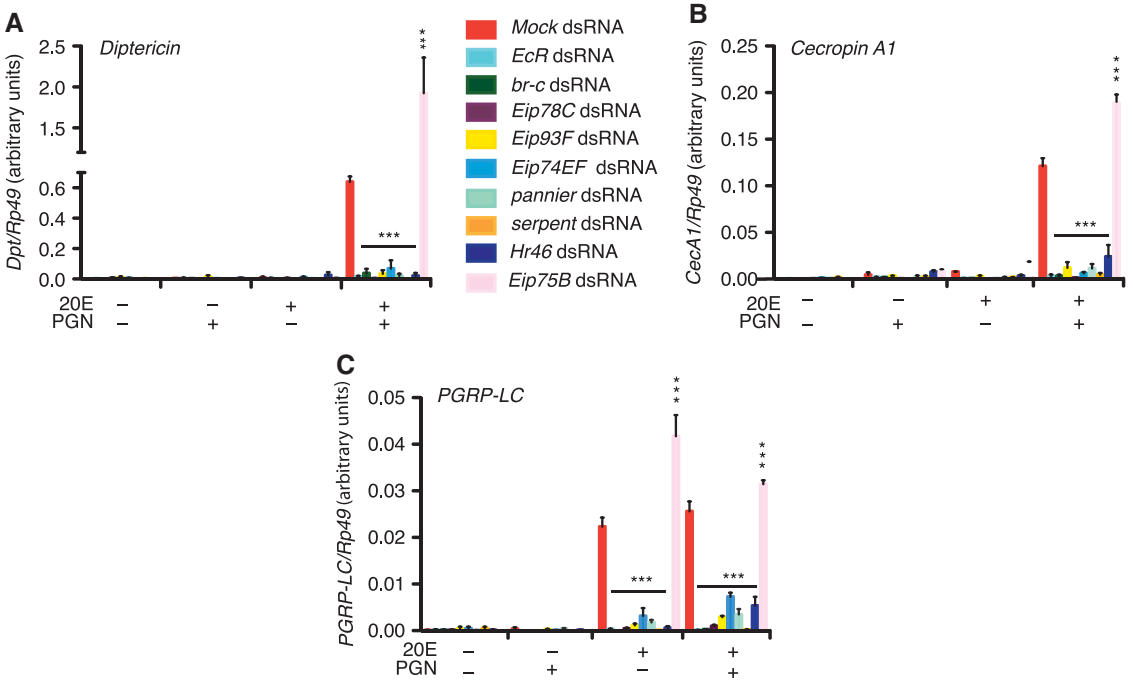

Figure 4 Classic ecdysone signalling pathway components regulate *PGRP-LC* expression and *AMP* gene induction. (**A** and **B**) qRT–PCR analysis of *Dpt* (**A**) or *Cecropin A1* (**B**) induction in S2* cells transfected with RNAi targeting for *EcR, br-c, Eip78C, Eip93F, Eip 74EF, Eip75B, Hr46, pnr, srp* or mock transfected. Cells with or without exposure to 20E for 24 h were then stimulated (or not) with PGN for an additional 6 h, as indicated. (**C**) The same RNA was analyzed for the expression of *PGRP-LC* by qRT–PCR. The mean and s.d. of three independent experiments is shown. *P* values were calculated by one-way ANOVA with Tukey's multiple comparison test, $***P<0.001$.

compared to *Yp1-GAL4* alone females (Figure 6D). Control males did not exhibit any of these phenotypes (Supplementary Figure S8D). On the other hand, when the negative regulator Eip75B was similarly targeted by RNAi, flies showed significantly improved survival, thus loss of Eip75B leads to elevated AMP levels and enhanced immune defense.

Several recent studies have identified genes that affect the ability of flies to survive microbial infections, without altering their ability to kill and clear the infecting pathogen, referred to as tolerance mechanisms rather then resistance mechanisms (Ayres *et al*, 2008; Schneider and Ayres, 2008). In order to determine whether depletion of *EcR*, *br-c*, *Eip78C*, *Eip93F*, *Eip74EF*, *Eip75B*, *Hr46*, *srp* or *pnr* in the fat body leads to a reduced resistance or tolerance, we measured the number of bacteria present in the fly from 0–48 h post infection with *Ecc15* (Supplementary Figure S10). The results show that flies depleted of *EcR*, *br-c*, *Eip78C*, *Eip93F*, *Eip74EF*, *Hr46*, *srp* or *pnr* exhibit significantly increased in bacterial loads at 48 h post infection (Supplementary Figure S10A), indicating that interference with ecdysone signalling affects resistance mechanisms in the context of *Ecc15* infection. On the other hand, *Eip75B* RNAi flies more rapidly cleared the infection, with significantly reduced bacterial loads at 24 h consistent with the higher levels of PGRP-LC and AMPs observed in these animals (Supplementary Figure S10A). Control male flies of the same genotypes showed little or no change in *Ecc15* clearance, as expected (Supplementary Figure S10B). We also measured bacteria clearance with another Gram-negative bacteria, *Enterobacter cloacae*, in flies depleted of *EcR*, *br-c*, *Eip78C* and *Eip75B* (Supplementary Figure S11). Similarly, clearance of this infection was also dependent on 20E signalling, indicating an affect on resistance.

Discussion

The steroid hormone ecdysone has a critical role in coordinating molting, metamorphosis and reproduction in insects (Riddiford, 1993). Previous studies have also indicated that 20E has profound effects on the *Drosophila* immune response, especially the IMD-signalling pathway, but the underlying molecular mechanisms have so far remained obscure (Meister and Richards, 1996; Flatt *et al*, 2008; Zhang and Palli, 2009). In S2* cells, we show here that 20E controls the IMD pathway by at least two distinct mechanisms (Figure 7). First, 20E signalling regulates the expression of the receptor PGRP-LC, thereby affecting all IMD signalling outputs. Ectopic expression of the PGRP-LC receptor is sufficient to support IMD signal transduction and immune-induced expression of some *AMP* genes, *i.e.*, *Cecropin A1*, *Attacin A* and *Defensin*, in the absence of this steroid hormone. On closer inspection of the data in Figure 3, it is apparent that these *AMP* genes display weak and variable, but detectable PGN-induced expression in the parental S2* cell line (gray bars) in the absence of hormone pretreatment. This suggests that the *Cecropin A1*, *Attacin A* and *Defensin* loci are primed to respond to PGN stimulation, with AMP expression depending only on the level of the PGRP-LC receptor and the ensuing activity through the IMD signal transduction system. In contrast, the *Diptericin*, *Metchnikowin* and *Drosomycin* loci are nearly unresponsive without hormone pretreatment, regardless of PGRP-LC expression, consistent with the notion that a second, distinct

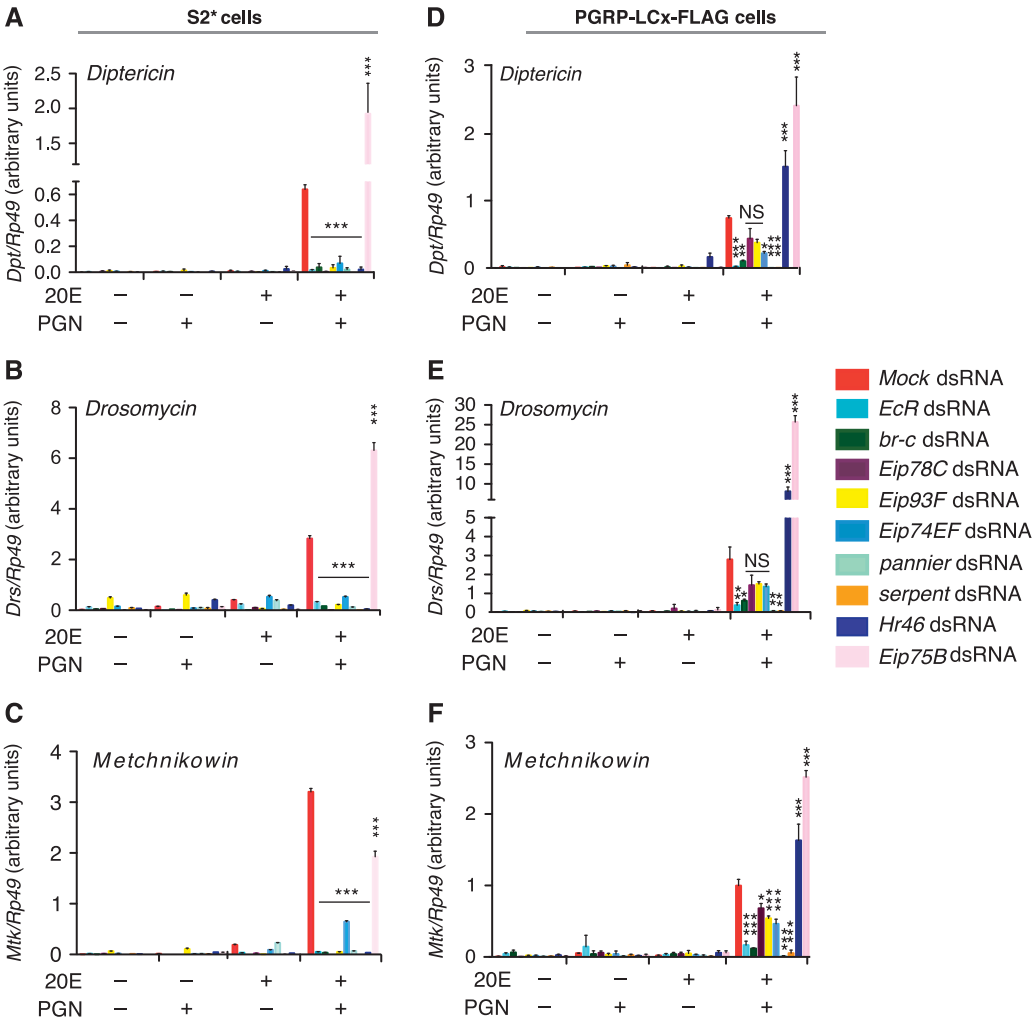

Figure 5 *EcR*, *br-c*, *srp* and *pnr* are critical for the PGRP-LC-independent hormonal control of *AMP* gene induction. qRT–PCR analysis of *Dpt*, *Drs* and *Mtk* expression in parental S2* cells (**A–C**) and PGRP-LCx-FLAG expressing cells (**D–F**) treated with RNAi targeting for *EcR*, *br-c*, *Eip78C*, *Eip93F*, *Eip74EF*, *Eip75B*, *Hr46*, *pnr*, *srp* or mock transfected. Cells were exposed or not to 20E treatment for 24 h and then stimulated (or not) with PGN for an additional 6 h, as indicated. The results shown represent the mean of three independent experiments and error bars are s.d. Statistical significance was determined by one-way ANOVA with Tukey's multiple comparison test, $*P<0.05$, $**P<0.01$, $***P<0.001$.

20E-dependent regulatory circuit directly controls these genes. This second mechanism of hormonal control is absolutely dependent on the EcR, BR-C, SRP and PNR transcription factors, with a lesser contribution from the other ecdysone-inducible transcription factors analyzed.

20E functions through binding to a heterodimeric nuclear hormone-receptor complex consisting of EcR and USP (Yao *et al*, 1993) that are both required for the effects of 20E on the IMD pathway (Flatt *et al*, 2008). Many of the immediate transcriptional targets of the EcR/USP complex are themselves transcription factors (Thummel, 1996), driving a complex hierarchy of transcriptional responses downstream of 20E. In addition to activating 'late' ecdysone-response genes, which include additional transcription factors, many of the 'early' ecdysone-inducible genes also regulate the expression of other 'early' response genes (Karim *et al*, 1993; Thummel, 1996; Lee and Baehrecke, 2001). In this study, we have analyzed 12 of the approximately 64 transcription factors that are induced by 20E at the 24 h time point in our RNA profiling data, while the analysis of the remaining 52 transcription factors await further investigation. Our RNAi-mediated depletion experiments show that seven of these factors (*br-c*, *Eip93F*, *Eip78C*, *Ep74EF*, *Hr46*, *srp* and *pnr*) have a critical role in the 20E-mediated support of IMD signalling in cells and in adult animals. The *in vivo* data further suggest that the ecdysone-signalling cascade regulates immune-resistance mechanisms during bacterial infection, in order to limit microbial growth.

Our results demonstrate that reduced 20E signalling can create severe immune deficiency, even in the adult fly where levels of 20E are quite low (Handler, 1982; Schwedes and Carney, 2012), thus underscoring the fundamental role of 20E signalling in promoting immunity in the adult animal.

Interestingly, the dGATAb factor SRP has been previously linked to the *Drosophila* immune response. In particular, robust induction of *Cecropin A1* in the larval fat body requires a GATA element in addition to Relish-binding κB sites (Kadalayil *et al*, 1997; Petersen *et al*, 1999; Tingvall *et al*, 2001). In addition to *Cecropin A1*, five other *AMP* genes (*Attacin A*, *Defensin*, *Drosomycin*, *Diptericin*

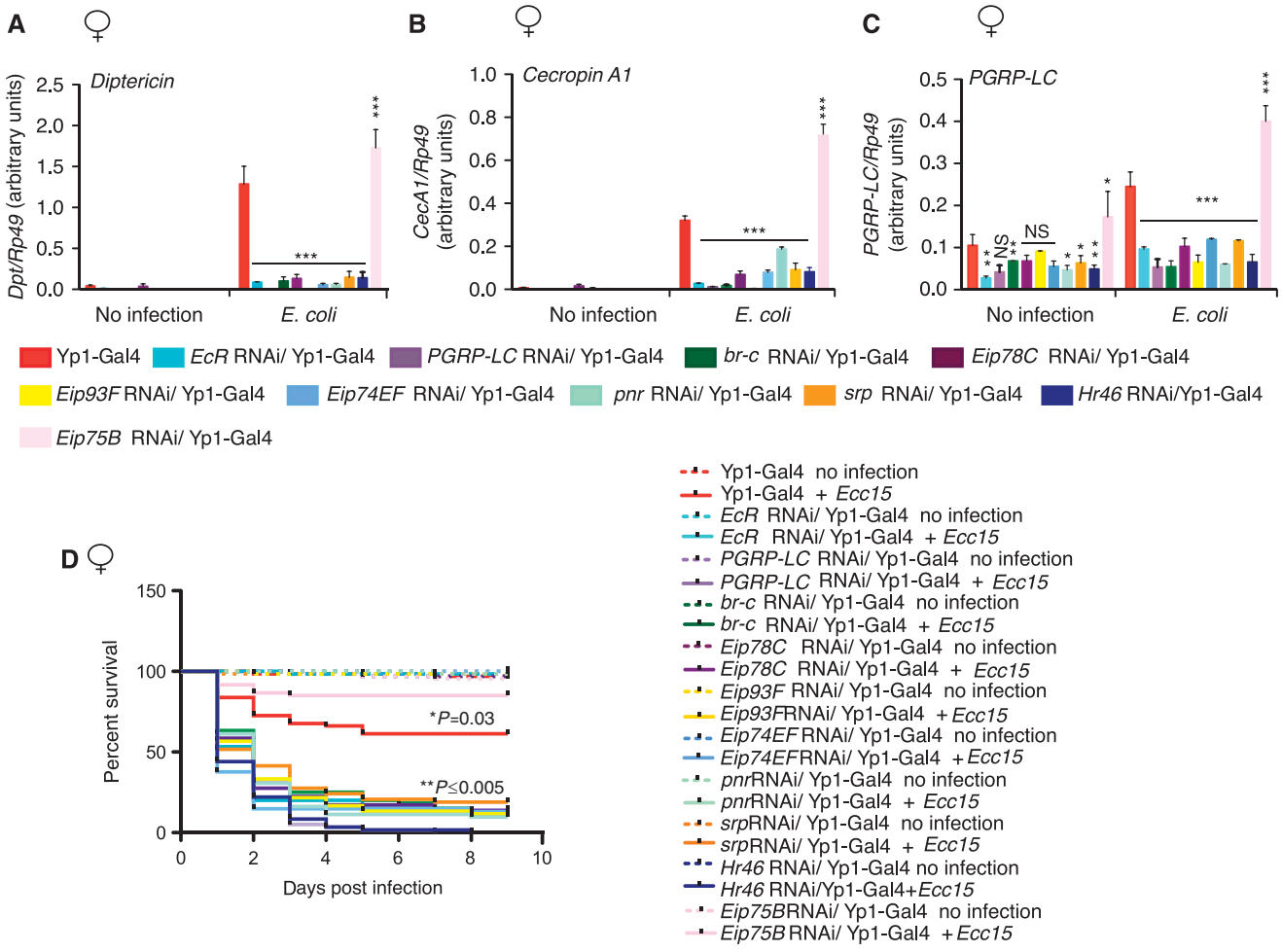

Figure 6 *EcR*, *br-c*, *Eip93F*, *Eip78C*, *Eip74EF*, *Hr46*, *pnr*, and *srp* knockdown causes immunodeficiency in adult flies. Real-time RT–PCR was used to analyze the expression of *Dpt* (**A**), *CecA1* (**B**) and *PGRP-LC* (**C**) in *EcR*, *br-c*, *Eip78C*, *Eip93F*, *Eip74EF*, *Eip75B*, *Hr46*, *pnr* or *srp* RNAi expressing flies before or 24 h after infection with *E. coli*. For all experiments, the *yolk protein 1 (Yp1)-GAL4* driver was used to express inverted-repeat RNAs specifically in the adult female fat body, and the *Yp1-GAL4* strain is presented as a control. *P*-values were calculated in comparison to *Yp1-GAL4* driver strain by one-way ANOVA with Tukey's multiple comparison test. $*P<0.05$, $**P<0.01$, $***P<0.001$, (**D**) Kaplan–Meier plot showing survival of these same lines after infection with *Erwinia carotovora carotovora 15*. Survival curves of uninfected animals of all genotypes, overlap and are shown as dashed lines. Statistical significance between the survival of infected RNAi flies and the control *Yp1-GAL4* strain were determined by a log-rank test and is equal or less then 0.005 for all comparisons, except for *Eip75B* RNAi, which survives better with a $P=0.03$. $n=60$ for all genotypes, and results are typical of at least three independent experiments.

and *Metchnikowin*) contain a common organization of regulatory elements: closely linked NF-κB and GATA-binding sites clustered near their transcription start site (Senger *et al*, 2004, 2006). Interestingly, *PGRP-LC* also contains a perfect GATA element, in the third exon, that could serve as a binding site for SRP and/or PNR. A direct repeat of NHR-binding elements, which could potentially bind EcR, USP, Hr46, or Eip75B, are also found in the third exon of *PGRP-LC*. Future studies will be required to evaluate, which of these factors, and/or other factors, directly control hormone-induced *PGRP-LC* expression.

While five early ecdysone-induced transcription factors (BR-C, Eip93F, Eip78C, Eip74EF and HR46) positively regulate the IMD pathway, Eip75B, another early ecdysone-inducible gene and a nuclear hormone receptor itself, acts as a negative regulator of the innate immune response. Consistent with this observation, Eip75B is known to repress some aspects of the 20E-induced regulatory network through its ability to heterodimerize and interfere with transcriptional activation mediated by another nuclear hormone receptor, HR46 (Thummel, 1997; White *et al*, 1997; Yamanaka and O'Connor, 2011). Interestingly, HR46 and Eip75B are homologs of ROR and Rev-Erb respectively, that regulate mammalian immune responses. RORα and γ are best known for their critical role in stimulating Th17 development in response to innate immune triggers, such as viral, bacterial, fungal and parasitic infections, promoting production IL-17, IL-21 and IL-22 (Bettelli *et al*, 2008; Yang *et al*, 2008; Jetten, 2009; van de Veerdonk *et al*, 2009), while REV-Erbα has been linked to the circadian control of innate immunity (Ramakrishnan and Muscat, 2006; Gibbs *et al*, 2012). Interestingly, Eip75B (and REV-Erbβ) utilize heme as a cofactor for the ligand-binding domain. In addition, the heme-E75B complex is repressed by the gases CO or NO, or by oxidation (Caceres *et al*, 2011; Johnston *et al*, 2011).

Taken together, these results suggest novel mechanisms for the regulation of insect immunity by both hormonal and environmental factors. Acting through EcR and a set of

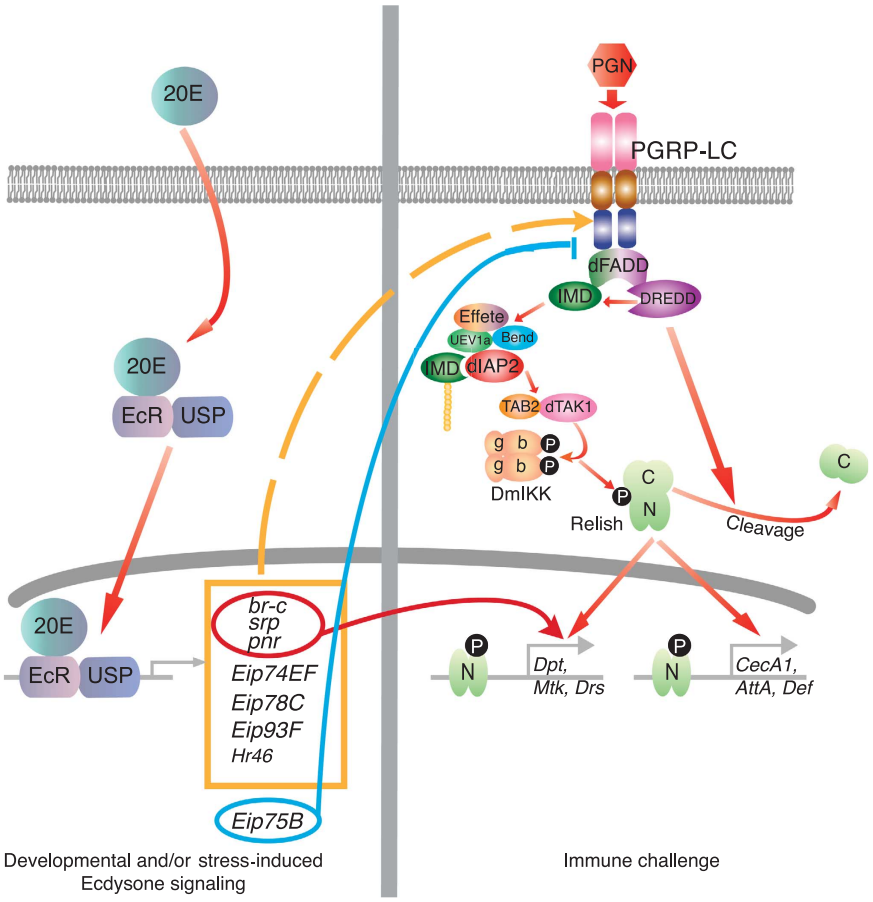

Figure 7 Model for 20E regulation of IMD innate immune signalling. 20E controls the IMD innate immune signalling by at least two distinct mechanisms. First, 20E regulates the expression of the peptidoglycan receptor *PGRP-LC*. This hormonal control involves several ecdysone-inducible transcription factors, including BR-C, Eip78C, Eip93F, Eip74EF, Eip75B, HR46, PNR and SRP. Through this steroid-mediated regulation of the key microbial sensor, immune induction of all *AMP* genes through the IMD pathway is tightly controlled by prior exposure to this hormone. Through a second mechanism, 20E further regulates the expression of a subset of *AMP* genes (*i.e.*, *Dipt*, *Mtk* and *Drs*), independent of its control of the receptor *PGRP-LC*. BR-C, SRP and PNR transcription factors are absolutely required for this *PGRP-LC* independent hormonal effect. On the other hand, the 20E-inducible nuclear hormone receptor Eip75B negatively regulates the IMD pathway, at least in part, by interfering with *PGRP-LC* expression.

downstream transcription factors, the steroid 20E potently primes the IMD pathway for a rapid response to infection. In addition, the 20E response also includes a self-limiting component, through the expression of the negative regulator Eip75B. However, Eip75B itself can be further modulated by various environmental factors. These modulators include the heme-mediated activation of E75B, (which is expected to further repress immune responses) or the CO/NO/oxidation-mediated inhibition of the E75B/heme complex, potentiating IMD responses. Interestingly, NO has been implicated in a systemic IMD response following a local oral infection or damage (Foley and O'Farrell, 2003; Wu *et al*, 2012), although the underlying mechanisms to generate or to respond to this gas remain unclear (Chakrabarti *et al*, 2012). The findings presented here suggest a potential mechanism whereby NO could enhance an IMD-mediated response by interfering with E75B. In addition, the heme-binding attributes of Eip75B were recently linked to the blood-meal triggered expression of the *vitellogenin* (*Vg*) gene in the mosquito *Aedes aegypti* fat body (Cruz *et al*, 2012), suggesting that this heme-responsive transcription factor may simultaneously stimulate oogenesis and block IMD signalling, especially in hematophagous insects.

While our current data clearly demonstrate a critical role for 20E signalling in the regulation of the *Drosophila* immune response, the underlying reasons for this hormonal control of immunity remain opaque. Two possible explanations readily come to mind. In *Drosophila* and other insects, 20E and the sesquiterpenoid juvenile hormone (JH) have critical roles in orchestrating the major transitions during development, with high levels of 20E and JH driving molting and 20E alone triggering pupation (Dubrovsky, 2004). In an earlier study, we showed that JH counteracts the IMD-potentiating effects of 20E (Flatt *et al*, 2008). Thus, the ability of 20E and JH to counter-regulate immune function may indicate that the fly sculpts its immune system into the most adaptive configuration for each life stage. The evolutionary pressures that might have created such a regulatory network are unclear, but it may relate to the different microbial threats that are commonly encountered by larvae, pupae or adults.

Another possible reason for tight hormonal control of the *Drosophila* immune response is inspired by mammalian physiology, where neuroendocrine modulation of the immune response is well-established. The main effectors of this neuroendocrine regulation of mammalian immunity are the glucocorticoids (Webster *et al*, 2002; Glaser and Kiecolt-

Glaser, 2005). Many stressors cause rapid activation of the hypothalamic–pituitary–adrenal axis, thereby initiating a hormonal cascade resulting in the systemic release of glucocorticoids, which in turn regulate the expression of innate immune and inflammatory genes through the glucocorticoid receptor. In adult flies, several studies have demonstrated that stress, for example induced by nutrient restriction, heat treatment or sleep deprivation, leads to increased levels of 20E (Rauschenbach *et al*, 2000; Terashima *et al*, 2005; Ishimoto and Kitamoto, 2010, 2011). Thus, it is conceivable that the regulatory network delineated in this study is part of a neuroendocrine-immune axis, whereby stress-induced elevation of the steroid hormone 20E drives elevated *PGRP-LC* expression and primes all the *AMP* genes, enabling a more robust immune response during times of stress. Although the direction of this regulation is opposite of that observed in mammals, where stress-induced glucocorticoids are best known to pharmacologically reduce the inflammatory response, several recent reports have clearly shown that glucocorticoids, when produced at physiological levels, actually induce the expression of innate immune receptors like TLR2 and NLRP3 (Shuto *et al*, 2002; Hermoso *et al*, 2004; Sakai *et al*, 2004; Busillo *et al*, 2011), very similar to the 20E-PGRP-LC axis demonstrated here. Thus, a profound but poorly understood conservation in the neuroendocrine regulation of innate immunity may exist in invertebrates and mammals.

Materials and methods

Microarrays

Affymetrix Drosophila 2.0 Chips were probed in triplicate with RNA isolated from untreated cells or cells treated with 1 μM 20-hydroxyecdysone (Sigma) for 24 h.

cDNA products were hybridized at the Brown University Genomics Core Facility to Affymetrix GeneChip Drosophila_2.0 Genome Arrays (3 replicate chips per treatment) (Li and Wong, 2001). The expression data were analyzed with dCHIP software. (http://biosun1.harvard.edu/complab/dchip/).

Affymetrix microarray data supporting the studies reported here can be found on the GEO database, series record number GSE46020.

Stable cell lines and cell culture

The FLAG-tagged PGRP-LCx construct was cloned into pRmHa3 vector, containing the copper-inducible MT promoter. The construct was then transfected into S2* cells in conjunction with pHs-Neo at a ratio of 50:1 and stable transfectants were selected with G418 (1 mg/ml). The YFP-Relish construct, cloned in pPacPL vector containing the actin promoter, was transfected into the stable cell line expressing the FLAG-tagged PGRP-LCx and into the parental S2* cells in conjunction with BM-IEG-hygromycin at a ratio of 50:1; stable transfectants were then selected with hygromycin (20 U/ml). *Drosophila* S2* cells were grown in Schneider's medium (Gibco) with 10% fetal bovine serum, 1% Gluta-MAX (Gibco), and 0.2% penicillin-streptomycin (Gibco) at 27°C.

Co-immunoprecipitation and immunoblotting assays

For immunoblotting analysis, PGRP-LCx-FLAG stable cells and parental S2* cells were split to 1.0×10^6/ml, treated or left untreated with 1 μM 20E (Sigma) for 24 h, and then stimulated with 100 μM copper sulphate for 3 h, when necessary, for increased expression from the metallothionein promoter. For immune stimulation, as indicated, some samples were stimulated for 10 min with 2 μg/ml PGN. The cells were lysed in lysis buffer (20 mM Tris at pH 7.6, 150 mM NaCl, 2 mM EDTA, 10% Glycerol, 1% Triton X-100, 1 mM DTT, NaVO4, glycerol 2-phosphate and protease inhibitors). Total protein extracts were separated by SDS–PAGE and transferred to PVDF membrane (Bio-Rad). Immunoprecipitations were performed with mouse anti-FLAG M2 (Sigma) or anti-IMD (Paquette *et al*, 2010) antibodies in lysis buffer. For immunoblot analysis, we used anti-FLAG-M2 (Sigma), anti-Ubiquitin (Santa-Cruz) or anti-IMD, anti-Ank-Relish (Stöven *et al*, 2003) and anti-phospho-Rel (Ertürk-Hasdemir *et al*, 2009) antibodies. The proteins were detected using HRP-linked anti-mouse IgG (GE Amersham) or anti-rabbit IgG (Bio-Rad) and ECL detection system (Thermo Scientific). Bands were visualized using a Fuji LAS-4000 cooled CCD camera/Dark Box, employing the Image Reader LAS-4000 v 1.1 software. The intensity of Relish protein bands shown in Figure 2 was measured using Multi-Gauge Imaging software and the percentage of Relish cleavage was calculated by dividing the intensity of the cleaved Relish protein by the total Relish intensity.

RNA and qRT–PCR

Total RNA from cultured cells or flies was isolated with the TRIzol reagent (Invitrogen) as previously described (Rutschmann *et al*, 2000) and then treated with DNase and re-extracted with phenol-chloroform. cDNA was synthesized using iScript cDNA synthesis kit (BioRad) and quantitative PCR analysis was performed on a DNA engine C1000 Thermal Cycler (BioRad), using SYBR Green (BioRad). The specificity of amplification was assessed for each sample by melting curve analysis and relative quantification was performed using a standard curve with dilutions of a standard. Quantified data were normalized to *Rp49* levels. To compare *AMP* gene expression between biological replicates, which always show a robust induction but with variations in the absolute level and amplitude, biological replicates were normalized relative to each other by setting the highest value in any given data set to 100%. Note that qRT–PCR analysis for PGRP-LC examines expression of all three splice-isoforms, with primers hybridizing to the common 5′ exons. In the cell-based experiments, samples were treated with 1 μM 20E for 24 h or left untreated and/or stimulated with 2 μg/ml PGN for additional 6 h prior to harvest for RNA extraction. For *in vivo* immune stimulation assays, adult flies were infected by pricking in the abdomen with a microsurgery needle dipped into a concentrated pellet of *E. coli 1106*. RNA was extracted 24 h later and assayed by qRT–PCR.

RNAi

RNAi (dsRNA) to *EcR*, *Eip74EF*, *Eip75B*, *Eip78C*, *Eip93F*, *br-c*, *Hr46*, *ERR*, *Hnf4*, *Hsf*, *luna*, *srp* and *pnr*, was produced using the T7 RiboMAX Express Large Scale RNA Production System (Promega). S2* cells were split to 1×10^6 cells per ml and incubated for 24 h at 27°C. RNAi (2 μg/ml) was then delivered by calcium phosphate transfection and cells were allowed to recover for ∼24 h at 27°C. Samples were then treated with 1 μM 20E for 24 h or left untreated, and/or stimulated with PGN (2 μg/ml) for additional 6 h before RNA isolation.

Confocal microscopy

For confocal microscopy, double-stable S2* cells expressing FLAG-tagged PGRP-LCx and YFP-Relish and single stables expressing the YFP-Relish were exposed or not to 1 μM 20E for 24 h and then plated on concanavalin A–treated 35 mm glass-bottomed culture. Samples were stimulated with 2 μg/ml PGN for 30 min when required. Cell membranes were stained with Cell Mask 10046 (Invitrogen), while the nuclei were stained with Hoechst 33342 (Invitrogen). The cells were visualized by fluorescence microscopy with a 63X objective on a Leica SP2 AOBS laser-scanning microscope. Images were generated by sequential scanning with 514 nm laser excitation and a 522–599 nm emission window for YFP, 649–666 nm laser excitation for Cell Mask C10046 and a 350–461 nm emission window for Hoechst 33342.

Fly strains and survival experiments

The *dominant temperature sensitive 3* (*DTS-3*) mutant and the *EcR* mutants: EcR^{A483T} and EcR^{NP5219}, as well as the control strains were obtained from Dr T Kitamoto (University of Iowa, Iowa City, Iowa, USA). *DTS-3* was induced in the wild-type strain Samarkand (Holden and Suzuki, 1973); EcR^{A483T} was generated by ethyl methane sulfonate mutagenesis on the *cn bw* background (Bender *et al*, 1997), while EcR^{NP5219} was generated by a *P*-element insertion in an *EcR* intron, on an iso(5) background (Yoshihara and Ito, 2000).

EcR, *br-c*, *Eip78C*, *Eip93F*, *Eip74EF*, *Eip75B*, *srp* and *pnr* RNAi were expressed in the adult female fat body by crossing: w [1118]; P{UAS-EcR^{GD1428}RNAi}v37058, w[1118]; P{UAS-$br\text{-}c^{GD4279}$RNAi}

v13705, w[1118]; P{UAS-*Eip78C*^{GD4135}RNAi}v10396, w[1118]; P{UAS-*Eip93F*^{GD4449}RNAi}v45857, P{UAS-*Eip74EF*^{KK109288}RNAi}VIE-260B, P{UAS-*Eip75B* ^{KK108982}RNAi}VIE-260B, w[1118]; P{*srp*^{GD12779}RNAi}v35578, P{*pnr*^{KK108962}RNAi}VIE-260B lines from the Vienna Drosophila RNAi Center (VDRC) (Dietzl *et al*, 2007) to the *Yp1-GAL4* driver line (w;P{GAL4-Yp1.JMR}20(yolkGAL4)) (Georgel *et al*, 2001) or in the fat body and hemocytes, using *C564-GAL4* driver line (Hrdlicka *et al*, 2002; Buchon *et al*, 2009). In all experiments 3–5 days old mated female flies were used and, as control, male flies with identical genotypes and/or GAL4 driver alone strains were included. Survival experiments were performed with 60 flies, following infection by pricking in the abdomen with a microsurgery needle dipped into a concentrated pellet *of Erwinia carotovora carotovora 15* (Basset *et al*, 2000; Zaidman-Remy *et al*, 2006). Surviving flies were transferred to fresh vials and counted daily for 9 days. Kaplan–Meier plots are presented and *P*-values were determined using unpaired *t*-test analysis.

Determination of CFU counts

To determine the bacterial load in flies at 0 h, 24 h and 48 h after infection with *Erwinia carotovora carotovora* 15 or *Enterobacter cloacae*, individual flies were homogenized in 200 µl and of phosphate-buffered saline. The homogenates were diluted in series (usually 10^{-1} to 10^{-3}), and the dilutions were plated on LB-Ampicilin or LB-nalidixic acid plates, as appropriate, and incubated overnight at 37°C for CFU counting.

Statistical analysis

Throughout, data are presented as mean values and error bars represent standard deviation. The variance and statistical significance were assessed using unpaired *t*-test or one-way ANOVA, with Tukey's post-test for multiple comparisons, as appropriate. For analysis of bacterial clearance assays, data were first normalized by logarithmic transformation and then the statistical significance was calculated by two-way ANOVA with gender and genotype as main effects, and paired comparisons to driver-alone control using Bonferroni post-test. Throughout, significance level is indicated as **** for $P<0.0001$, *** for $P<0.001$, ** for $P<0.01$, * for $P<0.05$ and NS for $P>0.05$.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Author contributions: FR, TF, MT and NS designed research; FR, TF, KA, KA, MT, TO, KO and EY performed research; FR, TF and NS analyzed the data; and FR and NS wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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