***Drosophila* Ras/MAPK signalling regulates innate immune responses in immune and intestinal stem cells**

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Immune signalling pathways need to be tightly regulated as overactivation of these pathways can result in chronic inflammatory diseases and cancer. NF- κ B signalling and associated innate immune pathways are crucial in the first line of defense against infection in all animals. In a genome-wide RNAi screen for modulators of *Drosophila* immune deficiency (IMD)/NF- κ B signalling, we identified components of the Ras/MAPK pathway as essential for suppression of IMD pathway activity, even in the absence of an immune challenge. Downregulation of Ras/MAPK activity mimics the induction of innate immune responses by microbial patterns. Conversely, ectopic Ras/MAPK pathway activation results in the suppression of *Drosophila* IMD/NF- κ B signalling. Mechanistically, we show that the Ras/MAPK pathway acts by inducing transcription of the IMD pathway inhibitor Pirk/Rudra/PIMS. Finally, *in vivo* experiments demonstrate a requirement for Ras/MAPK signalling in restricting innate immune responses in haemocytes, fat body and adult intestinal stem cells. Our observations provide an example of a pathway that promotes cell proliferation and has simultaneously been utilized to limit the immune response.

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Introduction

Drosophila is a well-established model for the study of innate immune responses and has led to the identification of evolutionarily conserved signalling pathways involved in both cellular and humoral aspects of host defense. Screens for *Drosophila* mutants in which the immune induction of antimicrobial peptides is abolished revealed two signalling pathways that regulate NF- κ B-dependent immune gene transcription: the Toll and immune deficiency (IMD) pathways (Lemaitre and Hoffmann, 2007).

The IMD pathway is activated by recognition of DAP-type (meso-diaminopimelic acid) peptidoglycans by the PGRP-LC and PGRP-LE receptors (Choe *et al*, 2002; Gottar *et al*, 2002; Takehana *et al*, 2004). Receptor activation brings about the association of the adaptor protein IMD, a homologue of human RIPK, with DmIcFADD and the apical caspase Dredd (Leulier *et al*, 2002; Naitza *et al*, 2002). *Drosophila* equivalents of the mammalian IKK complex, Ird5/IKK β and Kenny/IKK γ , phosphorylate and transactivate the NF- κ B homologue Relish (Rel) (Rutschmann *et al*, 2000; Silverman *et al*, 2000; Lu *et al*, 2001; Erturk-Hasdemir *et al*, 2009). Rel is cleaved, possibly by Dredd, whereupon the activated Rel domain translocates to the nucleus to activate target gene expression (Dushay *et al*, 1996; Hedengren *et al*, 1999). The transforming growth factor β -activated kinase 1 kinase and TAB are thought to activate the *Drosophila* IKK complex in an IMD- and possibly DmIcFADD-dependent manner (Kleino *et al*, 2005; Zhuang *et al*, 2006).

Genomic analysis of signal transduction, the identification of novel pathway components and the dissection of cross-regulatory mechanisms have advanced in recent years to offer more integrated models of how signalling pathways elicit coordinated responses (Fraser and Germain, 2009). Large-scale RNAi screens have been employed successfully in the identification of novel components and interconnections between signalling pathways in cultured cell lines as well as in model organisms (Boutros and Ahringer, 2008). Recently, several large-scale RNAi screens were conducted to find novel regulators of IMD signalling (Foley and O'Farrell, 2004; Gesellchen *et al*, 2005; Kleino *et al*, 2005). In an RNAi screen for regulators of IMD signalling, in cultured *Drosophila* cells of haematopoietic origin, several components of the Ras/MAPK signalling pathway were found to act as negative regulators (Gesellchen *et al*, 2005).

Most of our knowledge of Ras/MAPK signalling has been derived from work on receptor tyrosine kinases (RTKs), although Ras signalling can also be activated through other classes of membrane receptors. In *Drosophila*, RTKs initiate signalling by activation of the small GTPase Ras oncogene at 85D (Ras85D) (Simon *et al*, 1991). Activated Ras85D then triggers the sequential phosphorylation of the three MAPKs within the cascade; Pole hole (pHl, MAP3K/Raf1 homologue),

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which phosphorylates Downstream of raf1 (Dsr1, MAP2K1/MEK homologue) which then activates Rolled (RI, MAPK/ERK1/2 homologue) (Nishida *et al*, 1988; Tsuda *et al*, 1993; Brunner *et al*, 1994b). Activated RI modulates the expression of target genes through Ets transcription factors including the Pointed (Pnt) protein (O'Neill *et al*, 1994; Brunner *et al*, 1994a).

Here, we characterize the involvement of the Ras/MAPK pathway in the *Drosophila* innate immune response and show that active Ras/MAPK signalling is required for intrinsic suppression of IMD signalling in cultured cells and in all immune tissues examined *in vivo*, including haemocytes, fat body and the adult midgut. Concomitant with a role in immune suppression in the gut, we find that Ras/MAPK is also required for proliferation of intestinal stem cells (ISCs) and enteroblasts (EBs). We propose a model where Ras/MAPK signalling acts cell autonomously to repress PGRP-LC activity by modulating the expression of Pirk/Rudra/PIMS. Our results provide evidence for a novel interaction of Ras/MAPK and NF- κ B signalling that limits the strength of innate immune responses.

Results

Ras/MAPK pathway components are negative regulators of IMD signalling

We have previously described an RNAi screening approach to discover novel regulators of IMD signalling in SL2 cells (Gesellchen *et al*, 2005). Briefly, IMD pathway activity was monitored in SL2 cells, stimulated with heat inactivated *Escherichia coli*, using a luciferase reporter consisting of a

minimal promoter of *Metchnikowin*, fused to firefly luciferase (*mtk-luc*). The *mtk-luc* reporter contains Rel-binding sites that specifically respond to IMD/Rel but not to Toll signalling (Senger *et al*, 2004). The genome-wide RNAi screen identified 29 putative negative regulators of IMD signalling; among them eight were factors of the Ras/MAPK pathway (Figure 1A; Supplementary Table I). RNAi of the Ras/MAPK cascade components downstream of receptor kinase (*drk*), *Son of sevenless* (*Sos*), *Ras85D*, *phl*, *Dsr1*, *rl* and *pnt* resulted in increased *mtk-luc* reporter activity and z-scores ranging from 7.2 for *pnt* to 3.5 for *phl*. The z-score indicates the number of standard deviations an observed phenotype is above or below the mean observations in the screen. Consistent with the finding that knockdown of positive Ras/MAPK regulators enhanced IMD signalling, RNAi against *GTPase-activating protein 1* (*Gap1*), a negative regulator of Ras/MAPK signalling, led to significantly reduced IMD signalling activity (Gesellchen *et al*, 2005). The overrepresentation of Ras/MAPK cascade members (8 out of 29) among the candidate repressors in the screen strongly suggested a role for the Ras/MAPK cascade in the negative regulation of IMD signalling.

To confirm that the observed phenotypes were not caused by unintended knockdowns due to unspecific double-stranded RNA (dsRNA)-sequence matches in secondary genes (off-target effects) independent dsRNAs, and additional dsRNAs against Ras/MAPK signalling components, were used to validate the results from the screen. As shown in Figure 1B, RNAi of *platelet-derived growth factor* (PDGF)/*vascular endothelial growth factor* (VEGF) receptor (PVR)

Figure 1 Ras/MAPK signalling is required and sufficient for suppression of IMD signalling. (A) Identification of negative regulators of IMD signalling. Q-Q plot of normally distributed quantiles against screening result quantiles. A perfect fit to a normal distribution is represented by red line. Shown is the tail of negatively interacting dsRNAs. (B) Confirmation of Ras/MAPK pathway knockdown phenotypes in the IMD pathway luciferase reporter assay (*mtk-luc*) with independent dsRNAs in *Drosophila* SL2 cells stimulated with *E. coli*. (C) Depletion of Ras/MAPK components is sufficient to induce *mtk-luc* reporter activity in the absence of an immune stimulus. (D) Ectopic activation of Ras/MAPK pathway by expression of dominant active Ras (Ras85D^{V12}) suppresses the induction of IMD signalling by *E. coli*. Knockdown of *Ras85D* or factors acting downstream of Ras85D (*Phl*, *Dsr1*, *Pnt*) are able to rescue *mtk-luc* suppression. Data are shown as the mean (values above bars) and standard deviation of at least six replicates, and is representative of at least three independent experiments.

and the signalling components *Drk*, *Sos*, *Ras85D*, *Dsor1* and *rl* increased *mtk-luc* reporter activity after stimulation with *E. coli*. In contrast, depletion of *Gap1* significantly reduced *mtk-luc* activity. These results confirm and extend previous RNAi screen results implicating this pathway as a possible regulator of IMD signalling (Foley and O'Farrell, 2004; Gesellchen *et al*, 2005; Kleino *et al*, 2005; Bond and Foley, 2009).

Ras/MAPK signalling mediates intrinsic control of IMD signalling

Depletion of negative regulators of immune signalling can result in spurious immune responses in the absence of infection. We therefore asked whether disruption of Ras/MAPK pathway activity is able to induce IMD signalling in SL2 cells in the absence of immune stimulation. We monitored *mtk-luc* activity in cells depleted for members of the Ras/MAPK pathway in the absence of *E. coli* treatment. As shown in Figure 1C, depletion of Ras/MAPK signalling components resulted in an induction of the *mtk-luc* reporter induction without stimulation. This indicates that the IMD pathway in SL2 cells requires constitutive repression in the absence of immune stimulation to remain quiescent and that Ras/MAPK signalling is involved in this repression.

To further confirm a role of the Ras/MAPK pathway in the regulation of IMD signalling, we ectopically expressed dominant active Ras85D^{V12} in SL2 cells and monitored IMD activity. Activation of Ras/MAPK signalling by expression of Ras85D^{V12} strongly suppressed IMD pathway activity, upon stimulation, compared with the control (Figure 1D). Consistently, knockdown of pathway components at the level of Ras85D or downstream was able to rescue this phenotype. RNAi of *sos* was unable to reverse reporter repression while depletion of *Ras85D*, *phl*, *Dsor1* and *pnt*, in samples overexpressing Ras85D^{V12}, resulted in normal or increased reporter induction (Figure 1D).

Ras/MAPK signalling can be activated by several RTKs including epidermal growth factor receptor (EGFR), Torso, Sev (Sevenless) and PVR in *Drosophila* (Perrimon, 1994; Duchek *et al*, 2001; Cho *et al*, 2002). Our data suggested that the RTK PVR acts as the Ras/MAPK cascade initiating receptor in *Drosophila* SL2 cells. Pvf1–3 (PDGF- and VEGF-related factors 1–3) are proposed ligands for PVR and Pvf2 is described as a specific ligand for PVR in haemocytes (Munier *et al*, 2002; Bruckner *et al*, 2004). To examine whether PVR ligands are able to suppress IMD activity, Pvf1–3 were ectopically expressed in the *mtk-luc* reporter assay. To similarly occupy protein expression and ligand secretion, overexpression of Wingless (Wg) (Bartscherer *et al*, 2006) was used as a negative control. As shown in Supplementary Figure S1, overexpression of Pvf1, Pvf2 and Pvf3 all reduced *mtk-luc* activity while simultaneous RNAi of PVR in these cells was able to rescue this repression. RNAi of PVR in the control samples (control vector and Wg) resulted in ectopic *mtk-luc* activity, indicating that Pvf growth factors, signalling through PVR, are able to mediate Ras/MAPK repression of the IMD pathway in *Drosophila* haemocyte-like cells.

Modulation of Ras/MAPK signalling affects expression of immune responsive genes

To confirm that the RNAi phenotypes of Ras/MAPK components were not limited to the *mtk-luc* reporter assay, we

examined the induction of endogenous IMD pathway target genes, *Metchnikowin* (*Mtk*) and *Cecropin A2* (*CecA2*), by quantitative RT-PCR (qRT-PCR). SL2 cells were treated with dsRNA targeting *Dsor1* or *Gap1* before induction with *E. coli*. Knockdown of *Dsor1* enhanced the expression of *Mtk* and *CecA2* (Figure 2A and B). Conversely, RNAi of *Gap1* decreased *Mtk* and *CecA2* expression below GFP control levels. These results demonstrate that Ras/MAPK mediated repression of IMD signalling affects the expression of endogenous targets of the pathway.

To gain a more comprehensive view of the changes in IMD target gene expression upon modulation of Ras/MAPK signalling, and to elucidate whether Ras/MAPK signalling additionally interferes with other signalling routes, such as Toll immune signalling, we performed microarray expression profiling analysis. We compared transcript profiles from SL2 cells depleted for GFP, *Dsor1* or *Gap1* with samples treated with dsRNAs against *GFP* as a control. In addition, we compared the expression profiles of these cells in the presence or absence of *E. coli* stimulation. Transcriptome analysis revealed 39 genes that were significantly differentially expressed across all samples and could be grouped into three major clusters upon hierarchical clustering (Supplementary Figure S2, see Materials and methods). Hierarchical cluster analysis of differentially expressed genes in response to immune stimulation and to modulation of Ras/MAPK activity grouped all antimicrobial peptides together in one cluster. Figure 2C shows the cluster of genes enriched for known IMD pathway target genes including the *Attacins*, *Cecropins* and *Diptericin*. The IMD signalling component *Rel* and known negative regulators *PGRP-LF* and *Pirk/Rudra/PIMS* also clustered with the antimicrobial peptides. RNAi of *Dsor1* combined with *E. coli* treatment resulted in increased expression levels of this cluster of genes (Figure 2C). Conversely, upon *Gap1* RNAi IMD target gene induction was strongly repressed in the *E. coli* stimulated samples. *Gap1* depletion also induced upregulation of known direct target genes of Ras/MAPK signalling such as the negative Ras/MAPK regulator *sprouty* (Supplementary Figure S2). In summary, expression of several IMD target genes is linked to the levels of Ras/MAPK signalling in *Drosophila* SL2 cells.

Ras/MAPK signalling regulates the strength of the immune response

The expression of antimicrobial peptides is tightly regulated both in terms of magnitude of mRNA levels and kinetics of expression (Aggarwal and Silverman, 2008). Given that Ras/MAPK modulation affects IMD target gene induction, we next asked whether the altered levels of IMD target expression were due to a longer sustained immune response, that is loss of negative feedback on the pathway resulting in higher and/or prolonged expression of antimicrobial peptides.

We therefore examined the kinetics of expression of *Mtk* and *CecA2* by qRT-PCR in SL2 cells treated with dsRNA against *Dsor1* or *Gap1*. Upon *E. coli* stimulation, *Mtk* and *CecA2* display similar expression kinetics with a peak of expression at ~5 h after *E. coli* stimulation (Figure 2D and data not shown). Samples depleted for *Dsor1* displayed normal expression kinetics but a significant increase in the induction of *CecA2* (Figure 2D). Depletion of *Gap1* strongly suppressed the expression of *CecA2* as compared

Figure 2 Ras/MAPK pathway activity modulates the expression of antimicrobial peptides upon infection. The relative expression levels of antimicrobial peptides *Mtk* (A) and *CecA2* (B) were monitored by qRT-PCR, upon treatment with *E. coli*, in SL2 cells depleted for *Dsor1* and *Gap1*. RNAi of *Dsor1* results in increased expression, while *Gap1* RNAi results in decreased expression levels compared with the control (GFP dsRNA). (C) Cluster analysis of the transcriptional response of SL2 cells to *E. coli* treatment and Ras/MAPK modulation. Antimicrobial peptide and other IMD target genes are activated upon stimulation of SL2 cells with *E. coli*. Activation of Ras/MAPK signalling by depletion of *Gap1* potentially suppresses the induction of IMD target genes by *E. coli*. RNAi of *Dsor1* results in mild induction of target genes in the absence of stimulation and increased target gene expression upon stimulation compared with GFP control. In the cluster, red represents expression levels above the mean expression of a gene across all samples, white represents mean expression and blue represents expression lower than the mean (see colour scale at the bottom of the clustering). (D) Ras/MAPK activity alters the magnitude of IMD signalling but does not affect the kinetics of antimicrobial peptide expression. *CecA2* expression was monitored by qRT-PCR upon infection with *E. coli* in SL2 cells depleted for *Dsor1* and *Gap1*. Expression data for each dsRNA sample are normalized to the 0-h time point. Error bars represent the s.d. of three replicates. Data shown are representative of at least three independent experiments.

with the control. Similar results were observed with *Mtk* expression (data not shown), indicating that Ras/MAPK signalling is involved in restricting the magnitude of induction of antimicrobial peptides but not the kinetics of the immune response.

Ras/MAPK signalling represses IMD signalling in larval immune tissues in a cell autonomous manner

In order to assess the role of Ras/MAPK signalling in IMD pathway suppression *in vivo*, we first examined the larval immune response. We ectopically expressed components of the pathway specifically in the immune responsive organs of *Drosophila* third instar larvae using the Cg-Gal4 driver, which is expressed in the lymph gland, and circulating haemocytes and the fat body (Asha *et al*, 2003).

Third instar larvae were infected with a mixture of *E. coli* and *Micrococcus luteus* to induce the IMD and Toll pathways, respectively. Six hours after infection, haemocytes and fat body were isolated and the induction of the antimicrobial peptides, *Diatericin* (*Dpt*), an IMD pathway target, and *Drosomycin* (*Drs*), a Toll pathway target, was monitored by qRT-PCR. As shown in Figure 3A, overexpression of Ras85D^{V12}, PVR or Pvf2 resulted in robust repression of *Dpt* expression in the fat body, while *Drs* expression remained

comparable to the control (Supplementary Figure S3A). This phenotype is also seen in haemocytes isolated from infected larvae (Figure 3B; Supplementary Figure S3B). As the expression of *Drs* was not affected by Ras85D, PVR or Pvf2 expression, we can conclude that the general immune function of the fat body or haemocytes is not compromised. These results show that Ras/MAPK activity specifically inhibits IMD signalling *in vivo* in the larval haemocytes and fat body.

To confirm our observations in the larval fat body, we used a Diatericin-lacZ (*Dpt-lacZ*) reporter to monitor IMD pathway activity. We specifically depleted Ras85D in actin flipout clones in the larval fat body using a UAS-RNAi construct. As shown in Figure 3C and Supplementary Figure S4, in the absence of infection, RNAi of Ras85D results in a slight induction of the *Dpt-lacZ* reporter specifically in the Ras85D-depleted cells but not the neighbouring wild-type cells. Upon *E. coli* infection, *Dpt-lacZ* expression is increased in the Ras85D-depleted clones relative to the surrounding cells (Figure 3D; Supplementary Figure S4). This supports our observations *in vitro* that Ras signalling is required for intrinsic repression of basal IMD activity. Our results also suggest that the Ras pathway acts cell autonomously to repress IMD signalling.

Figure 3 Modulating MAPK signalling alters IMD pathway activity *in vivo* in the larval fat body and haemocytes. Ectopic activation of the MAPK pathway by overexpression of UAS-Ras85D^{V12}, PVR and Pvf2 with Cg-Gal4 (Cg>). *Dpt* expression was monitored by qRT-PCR in isolated fat body (A) or haemocytes (B) 6 h after infection with a mixture of *E. coli* and *M. luteus*. (A) Overexpression of Ras/MAPK components results in significant decreases in *Dpt* induction. *kenny* mutant flies (*key*¹) are included as a positive control. Mean values are shown above the bars (N = 12 for all genotypes, except *key*¹, N = 6). Data are representative of at least four independent experiments. (B) *Dpt* expression is also reduced in haemocytes overexpressing Ras85D^{V12} and Pvf2. Data are shown as *Dpt* expression levels relative to the w¹¹¹⁸ control. (C, D) Ras signalling modulates the IMD pathway in a cell autonomous manner. Depletion of Ras85D in flipout Gal4 clones in the larval fat body in the absence of infection (C) or upon infection with *E. coli* (D) results in upregulation of *Dpt-lacZ* specifically in the Ras85D-depleted clones but not neighbouring wild-type cells. AFG, actin flipout Gal4.

Ras/MAPK signalling modulates IMD signalling in adult flies

To rule out that Ras/MAPK mediated repression of IMD signalling occurs solely in haemocytes or the fat body in developing larvae, we also monitored antimicrobial peptide expression in adult flies. To this end, we used the Yolk protein 1 (Yp1-Gal4) driver (Georgel *et al*, 2001). This driver is expressed 3 days after eclosion in the female fat body thereby avoiding any effects on the development of the fat body. Four-day-old adult females were infected with either *E. coli* or *M. luteus*, and expression of *Dpt* and *Drs* was monitored 6 and 24 h after infection, respectively. Overexpression of Ras85D^{V12} resulted in significantly reduced *Dpt* expression while the expression of *Drs* was unaffected (Figure 4A; Supplementary Figure S5A). A stronger reduction in *Dpt* transcript levels was seen with overexpression of PVR. Flies containing Yp1-Gal4 or any of the UAS constructs on their own did not display changes in AMP expression demonstrating the specificity of our results (Supplementary Figure S6).

In order to examine the effect of downregulation of Ras/MAPK signalling on IMD pathway activity *in vivo*, we overexpressed the negative regulator Gap1 using Yp1-Gal4

and the EP insertion line Gap1^{EP45} (Rorth, 1996). Ras activity is regulated by GAPs, which stimulate the conversion of activated GTP-bound Ras to GDP-bound Ras, and thus function as a negative regulator of the pathway (Gaul *et al*, 1992). As shown in Figure 4B, overexpression of Gap1 resulted in a stronger induction of *Dpt* expression compared with the wild-type control, while *Drs* expression was not affected (Supplementary Figure S5B).

To confirm that Ras pathway downregulation results in increased AMP expression, we expressed UAS-RNAi constructs targeting Ras pathway components, using the tubGal4; tubGal80^{ts} system (tubulin-Gal4 combined with a temperature-sensitive Gal4 inhibitor, Gal80 (tubulin-Gal80^{ts})). This inducible system allows us to bypass lethality arising from loss of Ras signalling during development. Flies were raised at 18°C and then shifted to 29°C (3 days after eclosion) for 10 days to deplete Ras pathway components before infection with either *E. coli* or *M. luteus*. Expression of *Dpt* and *Drs* were monitored by qRT-PCR as above.

Upon RNAi of PVR, Dsor1 and Ras85D, we observe increased expression of *Dpt* (Figure 4C). In addition, we

Figure 4 Modulation of Ras/MAPK signalling alters IMD pathway activity *in vivo* in the adult fat body and gut. (A) Ectopic activation of the Ras/MAPK pathway in the adult fat body by overexpression of UAS-Ras85D^{V12} and PVR using Yp1-Gal4 (Yp1 >) results in a strong decrease in *Dpt* induction upon infection compared with the wild-type control ($N=12$ for all genotypes, except *key*¹, $N=6$). (B) Reduction of Ras/MAPK pathway activity through overexpression of Gap1^{EP45} results in increased induction of *Dpt* expression upon infection with *E. coli* (w¹¹¹⁸ and Gap1^{EP45}, $N=15$). (C) Depletion of Ras pathway components in adult flies using RNAi constructs and tubGal4^{ts} (tub^{ts} >) also results in increased *Dpt* expression upon infection. *kenny* mutant flies (*key*¹) and UAS-IMD-RNAi are positive controls (all genotypes $N=4$). *Dpt* expression was monitored by qRT-PCR in flies 6 h post-infection with *E. coli*. (D) In addition, modulation of Ras/MAPK activity using esgGal4^{ts} (esg^{ts} >) in the adult midgut affects *Dpt* induction. RNAi of Ras85D results in increased *Dpt* expression while overexpression of Ras85D^{V12} results in *Dpt* repression. Relative expression levels of *Dpt* were monitored by qRT-PCR in flies 18 h after infection with *E. carotovora carotovora* (*Ecc15*) (all genotypes $N=4$). Mean values are shown above the bars and data are representative of at least two independent experiments. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

observe increased expression of *CecA2* and *Mtk* in flies depleted for Ras signalling components (Supplementary Figure S7). These observations are in agreement with previous results showing that RNAi of PVR upregulates Attacin expression in adult flies (Bond and Foley, 2009). *Drs* expression was not affected in flies depleted for Ras pathway components (Supplementary Figure S5C). These results demonstrate that the Ras pathway specifically modulates IMD signalling activity *in vivo* but does not affect the ability of the fat body to produce antimicrobial peptides induced by Toll signalling.

Ras/MAPK signalling is required for immune suppression and stem cell proliferation in the *Drosophila* gut

The intestine constitutes one of the barrier epithelia against outside infection. The *Drosophila* midgut is of particular interest as a delicate balance between immune suppression against indigenous gut flora while a robust immune response against invading microbes is required. Recently, expression profiling and other studies of the immune response, specifically in the adult *Drosophila* midgut, has revealed the major role of the IMD pathway in antimicrobial peptide production

(Liehl *et al*, 2006; Ryu *et al*, 2006; Nehme *et al*, 2007; Buchon *et al*, 2009b). In addition, signalling pathways previously associated with the immune response have also been shown to be important for gut homeostasis. JNK and JAK/STAT signalling are required for gut epithelium renewal upon infection through ISC proliferation (Biteau *et al*, 2008; Cronin *et al*, 2009; Jiang *et al*, 2009; Buchon *et al*, 2009a).

To examine whether the modulation of Ras/MAPK signalling in the gut also affects the IMD mediated immune response, flies were orally infected with *Erwinia carotovora carotovora* (*Ecc15*), a Gram-negative bacterium. As expression of the Ras85-RNAi construct was lethal, we used the temperature-sensitive esgGal4^{ts} system (esgGal4 combined with tubGal80^{ts}) (Jiang *et al*, 2009). As shown in Figure 4D, esgGal4^{ts} flies expressing a Ras85D-RNAi construct showed increased *Dpt* expression upon infection. Conversely, overexpression of Ras85D^{V12} resulted in repression of *Dpt* expression consistent with our results in adult flies.

The adult *Drosophila* midgut is made up of mature enterocytes (ECs), EBs and ISCs (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). In order to examine at a cellular level which intestinal cell types are involved in Ras-mediated repression of IMD signalling, we monitored

Dpt-lacZ expression by immunofluorescence microscopy. *EsgGal4^{ts}* was employed to specifically deplete or overexpress Ras85D in ISCs and EBs (Figure 5). *MyoIA-Gal4^{ts}* (Jiang *et al*, 2009) was used to specifically target mature ECs. In adult guts of wild-type unchallenged flies, we observe low-level expression of the *Dpt-lacZ* reporter in ECs, consistent with our observations in the larval fat body (Figure 3C). RNAi of Ras85D using *esgGal4^{ts}* resulted in induction of *Dpt-lacZ* in the absence of infection (Figure 5B). Interestingly, *Dpt-lacZ* expression was observed in almost all the cells in the midgut, including ISCs, EBs and ECs. Depletion of Ras85D using *MyoIA-Gal4^{ts}* also resulted in induction of *Dpt-lacZ*, although the phenotype was weaker than that seen with *esgGal4^{ts}* and induction of *Dpt-lacZ* was only observed in ECs (Supplementary Figure S8B). As RNAi against Ras85D using *esgGal4^{ts}* is performed for 8 days before flies are infected, we hypothesize that RNAi in the ISCs and EBs also results in depletion of Ras85D in any differentiated daughter cells during the time course of this experiment. Upon infection with *Ecc15*, *Dpt-lacZ* is expressed in the mature ECs of wild-type midguts (Figure 5D). Depletion of Ras85D in ISCs and EBs results in increased *Dpt-lacZ* expression in all cell types (Figure 5E). RNAi of Ras85D specifically in ECs results in a weaker phenotype (Supplementary Figure S8E).

Conversely, overexpression of Ras85D^{V12} using *esgGal4^{ts}* resulted in repression of basal *Dpt-lacZ* expression in unchallenged flies (Figure 5C). In *Ecc15*-infected guts, high *Dpt-lacZ* expression is lost in most ECs and the staining pattern is more similar to that of unchallenged flies (Figure 5F). This repression is also observed using *MyoIA-Gal4^{ts}* after infection (Supplementary Figure S8F). These results provide further evidence of the role of Ras/MAPK in modulating IMD signalling in adult *Drosophila* immune tissues.

MAPK signalling has recently been shown to be required for proliferation of midgut progenitor cells in the developing larvae (Jiang and Edgar, 2009). As shown in Figure 5, we observe a decrease in the number of GFP-positive cells (ISCs and EBs) upon Ras85D-RNAi using *esgGal4^{ts}* (Figure 5A and B). Conversely, upon overexpression of Ras85D^{V12} GFP-positive cell counts increase, as previously observed (Apidianakis *et al*, 2009) (Figure 5A and C). To examine whether Ras signalling is also required for ISC proliferation in the adult midgut, we monitored EdU incorporation in flies depleted for Ras85D by RNAi or overexpressing Ras85D^{V12}, using *esgGal4^{ts}*, in the absence of infection (Figure 6). ISCs and EBs were visualized by co-expression of UAS-GFP. As shown in Figure 6A, in wild-type guts, 9% of cells were positive for EdU. Depletion of Ras85D resulted in fewer proliferating cells (5.5%) compared with wild-type guts (Figure 6B and D). Upon overactivation of the Ras/MAPK pathway, using UAS-Ras85D^{V12}, an increase in the number of EdU incorporating cells is seen (Figure 6C and D). This suggests that Ras/MAPK signalling is also required for cell proliferation in the adult midgut as well as in the midgut progenitor population during larval and pupal stages (Jiang and Edgar, 2009).

Ras/MAPK activity affects resistance to infection

We next asked whether Ras/MAPK pathway modulation would also affect resistance of *Drosophila* to infection with *Ecc15*. We therefore compared the survival of flies with altered Ras/MAPK activity upon infection by septic injury with *Ecc15*. As shown in Figure 7A, flies overexpressing

Ras85D^{V12} or PVR displayed decreased survival rates compared with the control (Yp1-Gal4/+). *Gap1* expression resulted in initial increased survival but did not offer significant long-term protection against infection. Similarly, depletion of Ras85D and *Dsor1* using *tubGal4^{ts}* results in initial increased survival but not significant long-term resistance to infection (Figure 7B).

We also infected flies with the fungus *Beauveria bassiana*, which elicits a mainly Toll pathway-dependent immune response. As shown in Supplementary Figure S9A, overexpression of Ras pathway components did not significantly alter survival rates compared with control, upon fungal infection, while *Dif^d* flies, which are defective in Toll-mediated immune signalling, are highly sensitive. Similarly, depletion of Ras pathway components by *tubGal4^{ts}* did not significantly alter survival rates upon fungal infection (Supplementary Figure S9B). However, the genetic background of *tubGal4^{ts}* renders flies more sensitive to fungal infection than Yp1-Gal4 flies (compare Supplementary Figure S9A and B). As a control, pricking flies with a sterile needle did not result in altered survival rates, demonstrating that altered Ras/MAPK signalling does not render the flies more sensitive to injury alone (see Supplementary Figure S9C and D).

Repression of IMD signalling by Ras/MAPK signalling occurs upstream or at the level of PGRP-LC

To determine at which level in the cascade Ras/MAPK activity suppresses IMD signalling, we performed an epistasis analysis in SL2 cells. We anticipated that knockdown of an IMD signalling component upstream of the point of intersection should not influence IMD pathway induction by Ras/MAPK knockdown. IMD signalling components were depleted by RNAi, while Ras/MAPK activity was decreased by RNAi of *Dsor1*. IMD pathway activity was monitored with the *mtk-luc* reporter. Co-depletion of *Dsor1* and *GFP* resulted in a strong induction of the reporter (Figure 8A). Depletion of PGRP-LC or any downstream component of the IMD pathway abolished the constitutive activation of *mtk-luc* induced by *Dsor1* knockdown. This result indicates that Ras/MAPK mediated suppression of IMD signalling acts upstream of or at the level of the PGRP-LC receptor.

We have shown earlier that all components of the Ras/MAPK cascade from PVR to *pnt* are required for the suppression of IMD signalling. To elucidate if this suppression is mediated through direct cross talk, between a Ras/MAPK pathway component and PGRP-LC, or by a gene target of the Ras/MAPK pathway we modulated Ras/MAPK activity in the presence of cycloheximide to block *de novo* protein translation. IMD pathway activity was monitored by qRT-PCR of *Mtk* and *CecA2* expression (Figure 8B; Supplementary Figure S10). *Drosophila* SL2 cells were incubated with dsRNAs against *GFP* or *Gap1* for 5 days and then pretreated with cycloheximide before stimulation with *E. coli*. As shown in Figure 8B, RNAi of *GFP* resulted in normal *CecA2* expression kinetics in the presence or absence of cycloheximide. As previously shown, activation of Ras/MAPK signalling through depletion of *Gap1* resulted in the repression of *CecA2* expression. However, in the presence of cycloheximide, this repressive activity was lost, resulting in a near normal *CecA2* expression profile. This result was also observed with *Mtk* expression (Supplementary Figure S10). The expression of *sprouty* in cycloheximide-treated cells was

Figure 5 Ras/MAPK signalling is required for intrinsic regulation of IMD signalling *in vivo* in the adult *Drosophila* midgut. (A–F) Transgenes were expressed in the adult ISCs and EBs using *esgGal4^{ts}* (*esg^{ts}* >). ISCs and EBs are marked by GFP expression (green) driven by *esgGal4^{ts}* and DNA stained with Hoechst (blue). *Dpt-lacZ* (red) was used as a reporter for IMD activity. (A) Wild-type midgut. (B) In the absence of infection, induction of *Dpt-lacZ* is observed upon expression of Ras85D-RNAi. (C) Overexpression of Ras85D^{V12} represses basal *Dpt-lacZ* expression. (D, E) Upon oral infection with *Ecc15* for 18 h, *Dpt-lacZ* is upregulated in Ras85D-depleted guts, (F) while ectopic Ras signalling suppresses IMD activity. Insets show high magnification images of ISCs.

Figure 6 Ras/MAPK signalling is required for proliferation of the ISCs. (A–C) Transgenes were expressed in the adult ISCs using *esgGal4*^{ts} (*esg*^{ts} >). ISCs and EBs are marked by GFP expression (green) driven by *esgGal4*^{ts} and DNA is stained with Hoechst (blue). EdU incorporation (red) was used to identify proliferating cells. (B) Expression of UAS-*Ras85D*-RNAi results in reduced numbers of EBs and ISCs, while expression of UAS-*Ras85D*^{V12} (C) results in increased proliferation. (D) Quantitation of EdU incorporation in the *Drosophila* gut upon modulation of Ras signalling. Data are shown as percentage of EdU-positive nuclei per midgut section (**P* < 0.05).

comparable to untreated cells indicating that cycloheximide did not affect Ras/MAPK pathway activity (data not shown). We therefore conclude that Ras/MAPK signalling controls IMD signalling through the expression of a repressor that inhibits IMD signalling at the level of PGRP-LC.

Ras/MAPK signalling activates the IMD negative regulator *Pirk*/Rudra/PIMS

Recent studies have identified *Pirk*/PIMS/Rudra as a negative regulator of IMD signalling. *Pirk* interacts directly with PGRP-

LC and PGRP-LE, and disrupts the interaction of PGRP-LC and IMD, thus interfering with IMD signalling (Aggarwal *et al*, 2008; Kleino *et al*, 2008; Lhocine *et al*, 2008).

To determine if *Pirk* could be the repressor induced by Ras/MAPK signalling, we examined the induction of *pirk* upon Ras/MAPK pathway modulation by qRT-PCR. As shown in Figure 8C, overexpression of *Pvf2* or *Ras85D*^{V12} in SL2 cells results in increased *pirk* expression. A similar phenotype is also observed in adult flies (Figure 8D). We therefore examined whether depletion of *pirk* in SL2

Figure 7 Ras/MAPK signalling affects resistance to Gram-negative bacterial infection. (A) Flies overexpressing Ras/MAPK components display decreased survival rates upon infection with *Ecc15*. (B) Flies depleted for Ras pathway components display partial resistance to infection with *Ecc15*. Data are presented in Kaplan-Meier plots and significance was analysed by log-rank test. Data are representative of three independent experiments.

cells or loss of *pirk* *in vivo* could rescue Ras/MAPK mediated repression of the IMD pathway (Figure 8E and F). SL2 cells were transfected with the *mtk-luc* reporter and Ras85D^{V12} overexpression construct and stimulated with *E. coli*. Depletion of *Dsor1* or *pirk* is able to rescue the repression of the *mtk-luc* reporter (Figure 8E). In flies, overexpression of Ras85D^{V12} in a *pirk* mutant background was unable to silence *Dpt* induction upon infection (Figure 8F). Finally, disrupting Ras/MAPK signalling by overexpression of Gap1 did not rescue the repression mediated by overexpression of Pirk. These results indicate that Pirk acts downstream of the Ras/MAPK pathway to repress signalling through PGRP-LC. Taken together, the epistasis analysis and the correlation of *pirk* expression with Ras/MAPK activation suggest that the Ras/MAPK pathway regulates the expression of Pirk, which then suppresses IMD signalling by disrupting the interaction of PGRP-LC and IMD.

Discussion

In this study, we have characterized a novel role for the Ras/MAPK pathway as a regulator of immune responses in *Drosophila*. Our results show that the Ras/MAPK pathway represses IMD pathway activity in either the presence or absence of an immune stimulus. Furthermore, the Ras/MAPK pathway represses IMD signalling both in *Drosophila* cell culture and *in vivo*. The *in vivo* requirement for Ras/MAPK in immunity was seen in developing larvae as well as adult fat body and midgut. The Ras/MAPK pathway acts to limit the maximum intensity of IMD pathway induction,

rather than the kinetics of the response, by controlling transcription of the key IMD inhibitor Pirk. We propose a model where the role of Ras/MAPK signalling in immunity is to limit the strength of IMD signalling during an infection and to prevent spurious immune induction in the absence of infection.

Recent work has shown that the intensity and duration of immune responses in *Drosophila* are under strict control (Aggarwal and Silverman, 2008). As in mammals, hyperactivated immune responses can be detrimental and efficient downregulation of signalling is critical for proper immune function, overall health and fecundity (Bischoff *et al*, 2006; Kim *et al*, 2007; Maillet *et al*, 2008). To insure effective control of the immune response, the innate immune pathways are modulated at multiple levels by negative regulators. Several of these factors are themselves target genes of these signalling pathways and thus mediate negative feedback regulation.

Pirk/Rudra/PIMS is an IMD pathway target that acts to mediate negative feedback during infection. While many negative feedback regulators are transcriptionally induced by the pathway they regulate, our results show that Pirk is also able to be induced by a different pathway, the Ras/MAPK pathway. Our results also show that Pirk is essential for the immune suppressive effect of Ras/MAPK activation, indicating that Pirk is the key point of integration of these two pathways. In adult flies, loss of *pirk* does not result in IMD induction in the absence of infection. Similarly, RNAi of Ras components in adult flies does not result in AMP induction as measured by qRT-PCR (data not shown). This is in contrast to our observations in the adult midgut.

Why the Ras/MAPK pathway is deployed in immune suppression remains unclear, but an intriguing possibility is that immune suppression may be particularly important in proliferating cells with high Ras/MAPK signalling. For example, our results show that, in the adult midgut, Ras/MAPK signalling has dual roles, promoting cell proliferation and also repressing IMD signalling. Aberrant activation of IMD signalling in ISCs may therefore be detrimental to gut homeostasis. In mammals, aberrant regulation of immune and inflammatory pathways are thought to be important contributors to inflammatory disease and cancer (Radtke *et al*, 2006; Fre *et al*, 2008; Milne *et al*, 2009).

In haemocytes, Ras/MAPK signalling elicited by PVR and its ligands, Pvf1–3, is involved in cell proliferation and migration during embryogenesis (Duchek *et al*, 2001; Cho *et al*, 2002; Munier *et al*, 2002; Bruckner *et al*, 2004; Ishimaru *et al*, 2004; Olofsson and Page, 2005). Pvf2 has a key role in haemocyte proliferation in larvae and PVR has been shown to control plasmacyte differentiation in the lymph gland (Munier *et al*, 2002; Jung *et al*, 2005). PVR has been found as a candidate negative regulator of IMD signalling in several independent RNAi screens for IMD signalling (Foley and O'Farrell, 2004; Kleino *et al*, 2005).

PVR has also recently been found in an RNAi screen for JNK signalling, and found to regulate AMP expression *in vivo* (Bond and Foley, 2009). In addition, it has been shown that Pvf2 and Pvf3 expression is induced in S2 cells upon stimulation with peptidoglycan, and that this induction was dependent on active JNK signalling (Bond and Foley, 2009). However, we did not observe significant upregulation of Pvf2 expression upon infection in haemocytes or the larval

Figure 8 Mechanism of Ras/MAPK signalling mediated repression of IMD signalling. (A) Ras/MAPK mediated suppression of IMD signalling acts upstream or at level of IMD receptor PGRP-LC. Depletion of PGRP-LC or downstream factors efficiently abolishes the induction of the IMD pathway upon loss of Ras/MAPK signalling (*Dsor1* RNAi). (B) Ras/MAPK mediated repression of IMD signalling requires *de novo* production of a repressor protein. *Drosophila* SL2 cells depleted for *Gap1* were induced with *E. coli* in the presence or absence of cycloheximide. Depletion of *Gap1* represses *CecA2* induction. In the presence of cycloheximide, the repressive phenotype caused by *Gap1* RNAi is reverted. (C) Ectopic activation of Ras/MAPK signalling results in increased *pirk* expression in *Drosophila* SL2 cells. *pirk* expression was monitored by qRT-PCR in SL2 cells upon Pvf2 and Ras85D^{V12} overexpression. (D) Expression of UAS-Ras85D^{V12} using Yp1-Gal4 (Yp1 >) results in increased expression of *pirk* *in vivo* as monitored by qRT-PCR. (E) Depletion of *pirk* rescues repression of IMD signalling mediated by Ras/MAPK activation. *Mtk-luc* activity was monitored upon Ras85D^{V12} overexpression in SL2 cells stimulated with *E. coli*. (F) Pirk acts downstream of Ras/MAPK signalling *in vivo*. Overexpression of UAS-Ras85^{V12} using Yp1-Gal4 (Yp1 >) in a *pirk*^{mut} (*pirk*^{EY00723}) mutant background does not result in reduced *Dpt* expression compared with *pirk*^{mut} alone. Similarly, overexpression of Gap1 (Gap1^{EP45}) is not able to overcome *Dpt* repression mediated by overexpression of Pirk (UAS-Pirk). Data are shown as mean (shown above the bars) and s.d. of at least four replicates, and are representative of at least two independent experiments. ***P* ≤ 0.005.

fat body, nor in whole adult flies *in vivo* (data not shown). It is therefore possible that induction of Pvf1–3 upon infection can act as a negative feedback loop on IMD signalling solely in the *Drosophila* gut *in vivo* and in S2 cells *in vitro*. In support of this, infection of the *Drosophila* gut induces expression of Pvf1–2 and the EGFR ligands Vein and Keren by approximately two- to three-fold (Buchon *et al*, 2009b). Pvf2 has also been shown to be upregulated in the ISCs of ageing flies and correlates with age-associated increases in ISCs and EB proliferation (Choi *et al*, 2008).

Further work is required to show whether upregulation of Pvf2 has an impact on the observation of immune senescence in ageing flies (Zerofsky *et al*, 2005; Ramsden *et al*, 2008).

Our results show that Ras/MAPK signalling mediates IMD repression in haemocytes and the adult midgut. Thus, it also has a dual role in promoting developmental proliferation and preventing spurious immune activation in immature immune cells of the fly. Aberrant immune signalling in the lymph gland has been shown to interfere with normal development (Schmidt *et al*, 2007) and could thereby destroy haemocytes

needed in a larval immune response and later during metamorphosis and adulthood. This raises the possibility that differential regulation of Ras/MAPK signalling may allow a degree of developmental control over immune responsiveness.

Immune suppression by Ras/MAPK signalling may also be important for the oncogenic effects of this pathway. Recently, it has been shown that overexpression of oncogenic Ras in the *Drosophila* ISCs causes intestinal dysplasia when flies are orally infected with virulent *Pseudomonas aeruginosa*, an opportunistic human gram-negative bacterium (Apidianakis *et al*, 2009). In agreement with this finding, we also observe increased disorder of the gut epithelium in Ras85D^{V12} overexpressing flies infected with *Ecc15* (see Figure 5). We speculate that Ras/MAPK mediated immune repression could be a contributing factor to the dysplasia seen in guts expressing oncogenic Ras through persistence of the infection coupled with the hyperproliferation of ISCs.

Concluding remarks

The IMD pathway is subject to multiple mechanisms of negative feedback to precisely control signalling activity in response to infection. We have shown that the magnitude of IMD responses can be further modulated by a second signalling pathway, the Ras/MAPK pathway, by having this pathway target transcription of an agent of negative feedback, Pirk. Ras/MAPK signalling could provide constitutive repression of IMD signalling in most tissues, and may do so more strongly in those tissues with the highest levels of signalling. Our observations provide an example of a signalling pathway that regulates cell proliferation that has simultaneously been utilized to limit the response of an innate immune pathway.

Materials and methods

Plasmid constructs

The *mtk-luc* firefly luciferase reporter and Rp128-RL Renilla luciferase reporter constructs have been described previously (Gesellchen *et al*, 2005; Bartscherer *et al*, 2006). To generate the Pvf2 expression plasmid, Pvf2 cDNA (clone RH40211) was subcloned into pAc5.1/V5-HisA (Invitrogen). pUAST-Pvf1 and pUAST-Pvf2 constructs were a gift from B Baum. The pMet-Ras85.V12 construct (a gift from S Katrav) consists of Ras85D.V12 under the control of the metallothionein promoter.

Cell culture and RNAi experiments

SL2 cells were maintained in Schneider's Medium (Invitrogen) (10% fetal bovine serum (PAA) and 1% penicillin/streptomycin (Invitrogen)) at 25°C. dsRNA synthesis, RNAi treatment and luciferase experiments were performed essentially as previously described (Muller *et al*, 2005). Complete primer and amplicon-sequence information can be found at <http://rna1.dkfz.de>. SL2 cells were transfected 24 h after dsRNA treatment using Effectene (Qiagen) and incubated at 25°C for 4 days. Heat inactivated *E. coli* was added to a final concentration of 200 µg/ml. Sixteen hours after stimulation, *mtk-luc* activity was measured with a Mithras LB940 plate reader (Berthold Technologies). To monitor Pirk expression, SL2 cells were seeded in 24-well plates and transfected with 1 µg pMET-Ras85D.V12 or pAc-Pvf2. Ras85.V12 expression was induced with CuSO₄ (500 µM) 6 h post-transfection and cells were harvested for RNA extraction 48 h later.

RNA Extraction and Quantitative RT-PCR

Total RNA was isolated using Trizol (Life Technologies) and reverse transcribed using Superscript III (Invitrogen). Quantitative RT-PCR was performed on a LightCycler (Roche), and transcript levels monitored using Universal Probe Library probes (Roche). Relative expression levels of target genes were calculated according to Pfaffl

(2001), using *rp49* as an internal control. Primer sequences and UPL probes in Supplementary Table II.

Time course analysis and cycloheximide treatment

SL2 cells were seeded in 24-well plates containing 10 µg dsRNA, induced with heat inactivated *E. coli*, and collected at appropriate time points by lysis in Trizol. Isolation of total RNA, reverse transcription and qRT-PCR analysis were performed as described above. For cycloheximide treatment, SL2 cells were incubated with 50 µM cycloheximide for 30 min before *E. coli* stimulation.

Expression profiling

For expression profiling on *Drosophila* Gene Array Affymetrix GeneChip arrays (Affymetrix) SL2 cells were treated with dsRNAs targeting GFP, Dsor1 or Gap1 for 4 days, induced with *E. coli* for 1 h, and RNA isolated as described above. cDNA synthesis was performed with the SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen). Generation and Biotin labelling of antisense cRNA was done using the High Yield RNA Transcript labeling Kit (Enzo Life Sciences). Image data were quantified using dChip software (<http://www.dchip.org>) (Li and Wong, 2001). For hierarchical clustering, the correlation distance metric and the centroid linkage method were used. Raw and normalized data, including detailed descriptions of the analysis methods used are available at ArrayExpress (<http://www.ebi.ac.uk/microarray-as/ae/>), Accession: E-MEXP-2145.

Drosophila strains

All stocks were maintained on standard medium at 25°C. Mutations are described in Flybase (<http://flybase.org>) and UAS-RNAi lines obtained from the Vienna *Drosophila* RNAi Center (VDRC; <http://stockcenter.vdrc.at/>). Mutants: *w¹¹¹⁸*, *key¹*, *Dif¹*, *pirk^{mut}*: *pirk^{EY00723}*. Gal4 lines used: *Cg-Gal4*, *Yp1-Gal4*, *w; esgGal4^{ts}*; *w; esgGal4 tubGal80^{ts} UAS-GFP*, *Myo1AGal4^{ts}*; *w; Myo1AGal4 tubGal80^{ts} UAS-GFP* (Jiang *et al*, 2009), *w; tubGal4^{ts}*; *w; tubGal4 tubGal80^{ts} UAS* lines used: *UAS-Ras85D^{V12}*, *UAS-PVR*, *UAS-Pvf2*, *Gap1^{EP45}*, *UAS-Dsor1-RNAi* (VDRC transformant ID 107276), *UAS-PVR-RNAi* (ID 105353). FRT: *AFG-Gal4*; *hsFLP*; *actin<stop>Gal4 UAS-GFP*. Reporters: *Diptericin-lacZ* (Reichhart *et al*, 1992). Transgenic RNAi lines *UAS-Ras85D-RNAi* (BKN28029), *UAS-IMD-RNAi* (BKN29863) and *UAS-Pelle-RNAi* (BKN22690) were kindly generated by Krystyna Keleman and Barry Dickson (landing site LS27, chromosome 2).

Larval infection

UAS transgenes were crossed to *Cg-Gal4* and progeny raised at 25°C. Third instar larvae were injected with a mixture of *E. coli* and *M. luteus* and incubated on agar plates for 6 h before fat body dissection and haemocyte collection. Haemocytes were collected on ice from at least 60 larvae per sample in PBS containing 1 × protease inhibitor cocktail (Roche). Fat body was collected from at least five larvae per sample. Actin flipout Gal4 clones, in the larval fat body, were generated by crossing UAS transgenes to *hsFLP*; *actin<stop>Gal4 UAS-GFP* flies and progeny raised at 18°C. Second instar larvae (72 h after egg deposition) were heat shocked for 30 min at 38°C. Fat bodies collected from wandering third instar larvae were then dissected in Schneider's medium and incubated with *E. coli* for 3 h to induce Dpt-lacZ expression before fixation.

Adult infection

UAS transgenes were crossed to *Yp1-Gal4* and progeny raised at 25°C. For infection experiments using *tubGal4^{ts}*, UAS-RNAi constructs were crossed to *w; tubGal4 tubGal80^{ts}* and progeny raised at 18°C. Flies were shifted to 29°C (3 days after eclosion) for 10 days to deplete mRNA targets. For septic injury, the thorax of flies was pricked with a needle dipped into a concentrated culture of *E. coli*, or *M. luteus*. Flies pricked with *E. coli* were incubated at 25°C for 6 h before collection; flies infected with *M. luteus* were incubated for 24 h at 25°C before collection.

Midgut infection

Adult gut infections were performed as previously described (Jiang *et al*, 2009). *Myo1AGal4^{ts}* or *esgGal4^{ts}* flies were crossed to UAS transgenes and progeny were raised at 18°C, and then shifted to 29°C (3 days after eclosion). For Ras85D^{V12} overexpression, flies were incubated at 29°C for 24 h while *UAS-Ras85D-RNAi* flies were incubated at 29°C for 10 days before infection. Oral infection with *E. carotova carotova* (*Ecc15*) was performed as previously described

(Buchon *et al*, 2009b) and flies collected 18 h after transfer to vials with contaminated food.

Antibodies and EdU incorporation

Diptericin-lacZ (*Dpt-lacZ*) was visualized using a rabbit anti-lacZ antibody (1:1000, ICL) and Alexa Fluor 594-conjugated secondary (1:1000, Invitrogen). GFP was visualized using a mouse anti-GFP-FITC-conjugated antibody (1:200, Biozol Diagnostics). DNA was visualized using Hoechst. For EdU incorporation experiments, the Click-iT EdU Alexa Fluor 594 Imaging Kit (Invitrogen) was used according to the manufacturer's instructions. Images were acquired on a Leica TCS SP5 confocal microscope.

Survival experiments

Flies were pricked with a needle dipped in a concentrated culture of *E. carotova carotova*, or with *B. bassiana* by shaking anaesthetized flies for 30 s in a Petri dish containing a sporulating fungal culture and incubated at 29°C. Thirty flies were infected per genotype and surviving flies counted every 24 h. Data are presented in Kaplan–Meier plots and *P*-values are calculated by log-rank test using GraphPad Prism.

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Supplementary data

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

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Conflict of interest

The authors declare that they have no conflict of interest.

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