**ird1** is a Vps15 homologue important for antibacterial immune responses in *Drosophila*

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Summary

The immune response-deficient 1 (*ird1*) gene was identified in a forward genetic screen as a novel regulator for the activation of *Imd* NFκB immune signalling pathway in *Drosophila*. *ird1* animals are also more susceptible to *Escherichia coli* and *Micrococcus luteus* bacterial infection. *ird1* encodes the *Drosophila* homologue of the Vps15/p150 serine/threonine kinase that regulates a class III phosphoinositide 3-kinase and is necessary for phagosome maturation and starvation-induced autophagy in yeast and mammalian cells. To gain insight into the role of *ird1* in the immune response, we examine how amino acid starvation affects the immune signalling pathways in *Drosophila*. Starvation, in the absence of infection, leads to expression of antimicrobial peptide (AMP) genes and this response is dependent on *ird1* and the *Imd* immune signalling pathway. Starvation, in addition to bacterial infection, suppresses the AMP response in wild-type animals and reduces the ability to survive *M. luteus* infection. Our results suggest that starvation and innate immune signalling may be intimately linked processes.

Introduction

Insects induce a battery of antimicrobial peptides (AMPs) in response to infection. Infection of *Drosophila melanogaster* with either bacterial or fungal pathogens induces expression of select AMP genes, indicating that there is specificity in the immune response (Lemaitre *et al*., 1997). Two signalling pathways, named *Imd* and *Toll*, have been shown to regulate a majority of the humoral (secreted) immune response in *Drosophila* (De Gregorio *et al*., 2002). These signalling pathways are highly conserved with mammalian innate immune pathways, as both are mediated by Toll-like receptors, peptidoglycan recognition proteins (PGRPs) and NFκB/Rel transcription factors (Hoffmann, 2003). The *Imd* pathway is characterized as being activated in response to Gram⁺ bacterial infection, and results in the expression of several antibacterial peptide genes including *Dipterican* (Hoffmann, 2003). Upon infection with Gram⁺ bacteria, the Gram⁺ diaminopimelic acid-type peptidoglycan is recognized by PGRP-LC (Choe *et al*., 2002; Gottar *et al*., 2002; Takehana *et al*., 2002; Leulier *et al*., 2003; Kaneko *et al*., 2004) and PGRP-LE (Takehana *et al*., 2002; 2004). These receptors transduce the signal via the death domain containing adaptor, *Imd* (Georgel *et al*., 2001), and the kinases, dTAK, a MAPKKK (Vidal *et al*., 2001; Silverman *et al*., 2003; Park *et al*., 2004), and the IκB kinase (IKK) complex of Ird5 (DmIKKββ) (Silverman *et al*., 2000; Lu *et al*., 2001) and Kenny (the IKKγ homologue) (Rutschmann *et al*., 2000a). dTAK is important for activation of both NFκB and JNK pathways (Silverman *et al*., 2003) and plays a key role in cross-talk between these two pathways (Park *et al*., 2004). IKK leads to phosphorylation of Relish (Silverman *et al*., 2000), a bipartite protein with Rel homology domains and an inhibitory ankyrin repeat domain, similar to mammalian p100 and p105 (Hedengren *et al*., 1999). Phosphorylated Relish is believed to become a substrate for the caspase Dredd that cleaves between the Rel domains and the inhibitory ankyrin repeat domains (Stoven *et al*., 2003). The N-terminal Rel portion of Relish is freed of its inhibition and retention in the cytoplasm, and translocates to the nucleus to activate transcription of antibacterial peptide genes.

The Toll pathway has been demonstrated to respond to fungal and Gram⁺ bacterial infection and results in the expression of the antifungal peptide gene *Drosomycin* (Lemaitre *et al*., 1996; Meng *et al*., 1999; Rutschmann *et al*., 2000b). Fungi and Gram⁺ bacteria are recognized by two different pathways that converge to activate the Toll ligand, Spätzle (Ligoxygakis *et al*., 1999; Ligoxygakis *et al*., 2002; Gobert *et al*., 2003). The processed form of Spätzle activates the receptor Toll (Weber *et al*., 2003; Hu *et al*., 2004). Downstream of Toll activation, the adaptors MyD88 (Krapfen) (Tauszig-Delamasure *et al*., 2002; Charatsi *et al*., 2003) and Tube activate Pelle, a serine/threonine kinase homologous to mammalian IRAK (Letou et al., 1991; Shelton and Wasserman, 1993; Grosshans *et al*., 1994). Cactus, the IκB homologue, is
then phosphorylated, ubiquitinated and targeted for degradation (Belvin et al., 1995; Bergmann et al., 1996; Reach et al., 1996). Upon degradation, Cactus can no longer inhibit the two NFκB transcription factors, Dorsal and Dif, and they translocate to the nucleus and regulate expression of immune target genes like the antifungal peptide gene, Drosomycin (Lemaitre et al., 1995; Meng et al., 1999; Rutschmann et al., 2000b).

Antimicrobial peptides were originally identified as haemolymph proteins that were induced in response to infection and had direct antimicrobial function (Boman, 2000). More recently, genome-wide microarray studies in Drosophila have indicated that the AMP genes are also induced in response to ageing, oxidative stress, autophagic cell death and starvation (Pletcher et al., 2002; Zinke et al., 2002; Lee et al., 2003; Landis et al., 2004). As these are conditions not normally associated with microbial infection, it suggests that the AMP signalling pathways are activated by other conditions, and AMPs may have a larger role in the physiology of the animal.

The ability to respond to infection requires energy and an immune response is tied to a concomitant activation of metabolic pathways (Lochmiller and Deerenberg, 2000). In mammals, pro-inflammatory cytokines or sepsis can trigger hypermetabolism which is important for the function of nutrient-demanding macrophages and for the large production of acute-phase proteins. Similarly, the adaptive immune response which involves clonal expansion of lymphocytes is also energy-expensive; mounting an antibody response comes with a significant energy cost (Demas et al., 1997) and activating a cellular response is dependent on the ability to mobilize glycolytic energy stores (Frauwirth et al., 2002). Because of the substantial energy cost required to sustain an immune response, the magnitude of an animal’s response can ultimately be limited by insufficient energy resources. Human populations with higher incidence of malnutrition tend to be more susceptible to infectious diseases (Chandra, 1996). Mice subjected to starvation are less likely to survive endotoxic shock (Faggioni et al., 2000) or chronic infections like tuberculosis (Chan et al., 1996).

The evidence indicates that there are interactions between nutrient-sensing pathways and immune responses (Matarese and La Cava, 2004). To date, not much is known about the molecular mechanisms linking nutrient levels to immune signalling – the evidence to this point has been largely correlative. In Drosophila, nutrient sensing occurs through the insulin and the target of rapamycin (TOR) pathways (Britton et al., 2002; Scott et al., 2004). These pathways are highly conserved in animals and have been primarily studied for their effects on growth. Insulin signalling is a hormone-based system which regulates cellular and organismal growth (Britton et al., 2002). The TOR pathway responds to nutrient levels to regulate protein synthesis and cell growth (Oldham et al., 2000; Zhang et al., 2000). During starvation, inhibition of the TOR pathway can induce autophagy, a process by which the cell recycles unwanted proteins and organelles to generate amino acids for other purposes (Scott et al., 2004). Downstream of TOR signalling, a phosphoinositide 3-kinase (PI3K) complex consisting of the class III PI3K, Vps34 and its regulatory partner/receptor subunit, Vps15 or p150, a serine-threonine kinase (Stack et al., 1995), are important components of the cell machinery to initiate autophagy (Petiot et al., 2000). This PI3K complex is also important for later events in endocytosis and phagocytosis in mammalian cells (Fratti et al., 2001; Futter et al., 2001; Vieira et al., 2001), and for the sorting of proteins and vesicles to the vacuole (lysosome equivalent) in yeast (Stack et al., 1995), so it is likely that the complex acts at a common key regulatory point for several cellular trafficking events.

We have identified the immune response deficient 1 (ird1) gene as a novel regulator affecting both the Imd and Toll pathways. ird1 mutants are unable to activate the Imd pathway, but have phenotypes that resemble constitutive activation of the Toll pathway. ird1 encodes the Drosophila homologue of the Saccharomyces cerevisiae Vps15 serine/threonine kinase known for its role in endocytosis, phagocytosis and starvation-induced autophagy in yeast and mammalian cells (Petiot et al., 2000; Fratti et al., 2001; Futter et al., 2001; Vieira et al., 2001). In exploring how a defect in one of these cellular processes might impact an innate immune response, we confirm the observation that amino acid starvation in the absence of infection induces AMP responses (Zinke et al., 2002). The starvation-triggered induction of these AMP responses requires ird1 and the Imd signalling pathway. The use of shared signalling pathways in both starvation and immune responses may provide a molecular mechanism for the regulation of innate immune responses in the context of nutrient control.

Results

ird1 is important for antibacterial immune responses

In an effort to identify novel components of the Imd anti-bacterial pathway, we screened for ethylmethane sulfonate-induced mutations on the third chromosome that impaired the induction of a Dipterocin–lacZ reporter gene in third-instar larvae upon infection with Escherichia coli (Wu and Anderson, 1998; Wu et al., 2001). Two mutant alleles of ird1 were identified in the original screen. Five more ird1 alleles were identified in subsequent genetic screens for mutations that failed to complement the lethality of the original alleles. These five ird1 alleles
also fail to complement the original alleles for the induc-
tion of the \textit{Diptericin–lacZ} reporter gene. We chose to study \textit{ird1} from among the 14 genes identified in the
screen because the mutation appeared to affect both the
\text{Imd} and \text{Toll} signalling pathways. Quantitative real-time
PCR analysis demonstrated that \textit{ird1} mutants failed to
induce the antibacterial peptide gene \textit{Diptericin}, which
confirms the \textit{Diptericin–lacZ} result from the screen
(Fig. 1). \textit{Diptericin} is considered to be a specific target
gene of the \text{Imd} pathway, because its induction is not
affected by \text{Toll} pathway mutations (Hedengren \textit{et al.}
1999; Meng \textit{et al.}, 1999; Rutschmann \textit{et al.}, 2000b). Our
result therefore suggests that \textit{ird1} is acting in the \text{Imd}
pathway (Fig. 1). We found that \textit{Drosomycin}, the target
gene specifically activated by the \text{Toll} pathway, was con-
stitutively expressed at a high level in \textit{ird1} mutants. This is
the opposite phenotype to that seen for \textit{Diptericin}. Con-
stitutive \textit{Drosomycin} expression has been observed in
mutants that activate the \text{Toll} pathway, such as \text{Toll^{10b}}
gain of function) mutants and \text{cactus} (loss of function)
mutants (Lemaitre \textit{et al.}, 1996).

We were interested in assaying other AMP genes
because they are regulated to some degree by both the
\text{Imd} and \text{Toll} pathways. The opposing effects \textit{ird1} has on
the two pathways appeared to affect the expression levels
of \text{Attacin}, \text{Defensin} and \text{Drosocin}. We saw differences in
\textit{ird1} alleles’ effects on these AMPs making it difficult
to draw conclusions. In \textit{ird1} animals, the induction of these
AMPs in response to infection is less than that of wild
type, similar to the effect of \textit{ird1} on \textit{Diptericin}. But the
uninduced level of these AMPs is also higher than wild-
type uninduced levels, suggestive of the effect of \textit{ird1} on

Fig. 1. \textit{ird1} affects antimicrobial peptide (AMP) gene expression. Shown are the expression levels in wild type (OR), \textit{ird1}\textsuperscript{1}, \textit{ird1}\textsuperscript{2} and \text{cact} (\text{cact}^{255}/\text{cact}^{255}) in uninfected or infected with \textit{E. coli} for 2 h larvae as measured by quantitative real-time PCR (Q-RT-PCR). \textit{Diptericin}
expression is reduced in \textit{ird1}\textsuperscript{1} and \textit{ird1}\textsuperscript{2} as compared with wild type (OR) in response to \textit{E. coli} infection for 2 h. \textit{Drosomycin} was constitutively expressed in the mutants. We see variability in the expression of the other AMPs in the \textit{ird1} alleles, ranging from low (\text{Attacin}, \text{Defensin},
\text{Drosocin}) to wild-type (\textit{Cecropin A}, \textit{Metchnikowin}) expression levels. The data suggest that the regulation of some of these other AMPs may
be impaired. In contrast to the \textit{Diptericin} and \textit{Drosomycin} AMP genes, the combinatorial contributions of the \text{Imd} and \text{Toll} pathways in the
regulation of the other AMPs are not as well characterized. \textit{cactus} (\text{cact}^{255}/\text{cact}^{255}) mutants which also constitutively express \textit{Drosomycin} were
examined for comparison. The expression levels have been normalized to \text{Rp49} levels and wild type uninfected has been set at 1 for
comparison between experiments. The experiments were performed in triplicate and the error bars indicate standard deviation. One-tail \textit{t}-tests
were used to compare wild type infected and uninfected to the corresponding AMP levels in the \textit{ird1} or \text{cactus} mutants. \textit{P}-values < 0.05 were
deemed significant and marked with asterisks.

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In general, the effect the ird1 mutation had on these AMP genes appeared to be more dramatic than the phenotype seen in cactus mutants. We found that the AMPs Metchnikowin and Cecropin were still inducible to near wild-type levels in ird1 mutants, indicating that ird1 does not have a non-specific effect on the induction of all the AMP genes. This AMP phenotype differed from other mutations identified from the original genetic screen that had non-specific effects on the fat body tissue, e.g. scribble/ird15, and resulted in decreased expression of all the AMPs examined (Wu et al., 2001).

To determine where ird1 acts in the Imd signalling pathway, we used genetic epistasis analyses. The Imd pathway can be activated in the absence of infection by ectopic expression of the Imd pathway components; these gain-of-function mutations are placed in combination with loss-of-function mutations affecting the pathway, and this can be used to determine the order of the components. The UAS-GAL4 expression system from yeast has been used as a heterologous expression system in Drosophila, and transgenic fly lines that express the GAL4 transactivator in different tissues are widely available (Brand and Perrimon, 1993). We confirmed that the c564-GAL4 driver [that drives expression of UAS-promoted genes in the fat body, lymph glands and other tissues (Harrison et al., 1995)] can lead to constitutive Drosomycin expression. Df(2L)J4 is a small deficiency that takes out both the Dif and dorsal genes (Meng et al., 1999). Mi, M. luteus.

**Fig. 2.** Genetic epistasis to place ird1 in the Imd and Toll pathways. Double mutant combinations were generated for epistasis analyses. Shown are the relative fold changes of Dipterican expression as measured using Q-RT-PCR in UAS-PGRP-LE/c564-GAL4 (set at 100) compared with UAS-PGRP-LE/c564-GAL4; ird1+/ird1+. Similar mutations combinations were generated with the UAS-imd, UAS-Dredd, UAS-dTak. For the Toll pathway, Drosomycin expression is shown in wild type (OR) uninfected (−) and infected (+) with M. luteus, in ird1 (ird1+/ird1+) uninfected and infected with M. luteus. To determine where ird1 was relative to other Toll pathway components, double mutants with spz (ird1+/spz+/ird1+/spz−) and Dif (Dif/Dif(2L)J4; ird1+/ird1+) were generated and then assessed for their ability to suppress the constitutive Drosomycin expression. Df(2L)J4 is a small deficiency that takes out both the Dif and dorsal genes (Meng et al., 1999). Mi, M. luteus.
in which the Imd signalling is occurring. A formal alternative is that ird1 may be acting in a pathway parallel to the Imd pathway in a complex manner.

To examine where ird1 was acting in the Toll pathway, we determined whether Toll pathway mutations could suppress the constitutive Drosomycin expression in ird1 mutants. A mutation in spätzle (spz) was able to suppress the Drosomycin expression. As Spätzle is the ligand for Toll (Weber et al., 2003; Hu et al., 2004), it suggests that ird1's effect on the Toll signalling pathway may be upstream of the fat body AMP response. We next examined the epistasis of ird1 relative to Dif. Dif is downstream in the Toll pathway and important for the regulation of Drosomycin expression in larvae. We found that a mutation in Dif was able to suppress the constitutive expression of Drosomycin in ird1 animals (Fig. 2). This suggests that the constitutive Drosomycin expression seen in ird1 animals is due to activation of the Toll pathway.

The AMP signalling pathways are often used to assess activation of the immune response, but a more meaningful measure of a functional immune response is the animal's ability to withstand bacterial infection. The effects of an ird1 mutation on survival of a bacterial challenge was tested using the Gram + bacteria, E. coli and the Gram − bacteria, Micrococcus luteus. These bacteria are not pathogenic to wild-type Oregon R flies (Fig. 3) and are often used to assay alterations in the Imd and Toll pathways respectively. We find that ird1 animals are significantly more susceptible than wild type to both E. coli (P = 0.03) and M. luteus (P = 0.0027) infection. The E. coli effect is consistent with the loss of Imd pathway function. The M. luteus susceptibility is counterintuitive as the Toll pathway is believed to be important for fighting a Gram − bacterial infection. However, our results indicate that constitutive activation of the Toll pathway is not protective against Gram + bacteria, and instead makes the animals more susceptible to infection.

The ird1 gene encodes the Drosophila homologue of the Vps15 kinase

Recombination and deficiency mapping localized the ird1 gene to the 85C1;85D8 interval on the third chromosome. Using male recombination mapping relative to P-elements in the region, ird1 mapped to the right of EP473 that was inserted in the D1 gene (Fig. 4A). From sequencing the candidate genes in the region in the ird1 alleles, we identified CG9746, a serine/threonine kinase with WD40 repeats as a good candidate for ird1. Five out of the seven ird1 alleles have mutations that result in premature stop codons in CG9746 that would lead to truncated proteins (Fig. 4B).

To verify that mutations in CG9746 were the cause of ird1 phenotypes, we generated transgenic flies expressing the ird1 gene under the control of an inducible promoter (Brand and Perrimon, 1993). Expression of this gene (P[w+, UAS-ird1]) in the ird1 mutant animals was sufficient to rescue the lethality and the AMP phenotype, confirming that CG9746 is the ird1 gene. The P[w+, UAS-ird1 PA10-68] insertion line in the absence of a GAL4 driver resulted in sufficient expression of the transgene for rescue of the ird1 phenotypes so this was used for the rescue experiments (Fig. 4C).

ird1 encodes the Drosophila protein most similar to yeast Vps15, a gene necessary for targeting proteins to the vacuole, the yeast equivalent of the lysosome (Herman et al., 1991). VPS15 is a serine/threonine kinase that acts as the regulatory subunit to Vps34, a class III
critical for endocytosis and starvation-induced autophagy in yeast and mammalian cells (Petiot et al., 2000; Fratti et al., 2001; Futter et al., 2001; Vieira et al., 2001). Our finding that the Drosophila Vps15 homologue plays a role in regulating NFκB signalling pathways is new. In an attempt to bridge the known function of Vps15 with this novel immune function, we explored whether starvation-induced Imd signalling pathway are important in a starvation-induced response. A whole genome microarray analysis in Drosophila first demonstrated that the AMP gene Drosomycin is induced by starvation.
in second-instar larvae subjected to amino acid and sugar starvation (Zinke et al., 2002). For our experiments, we used an amino acid starvation paradigm that had been demonstrated to affect the nutrient-sensing TOR pathway in the fat body of third-instar larvae (Scott et al., 2004). This starvation response has been better characterized and occurs in the same stage and tissue that we use for observing the ird1 immune phenotypes. We confirmed the initial microarray results by observing that a Drosomycin-green fluorescent protein (GFP) reporter is strongly induced at 3 h in amino acid-starved L3 animals (data not shown). The induction of the Drosomycin-GFP after amino acid starvation was more consistent from animal to animal, than the induction we typically observe with bacterial infection. We next examined the effect of amino acid starvation on the expression of the other known AMP genes by quantitative real-time PCR. Amino acid starvation significantly induced Diptericin, Drosocin, Drosomycin and Metchnikowin independent of infection (Fig. 5A).

**Fig. 5.** Effects of amino acid starvation on antimicrobial peptide immune phenotypes. 
A. In wild-type (OR) larvae, starvation (S) in the absence of infection (−) significantly induces the expression of Diptericin, Drosocin, Drosomycin and Metchnikowin as measured by Q-RT-PCR; shown are wild-type larvae either not starved (NS), starved (S) for 3 h, and uninfected (−) or infected with *E. coli* (+) for an additional 3 h. The induction of Attacin, Cecropin, Defensin and Drosocin is significantly lowered if the animals have been starved before infection.

B. Both ird1 and the Imd pathway are required for the starvation-induced expression of Diptericin and Metchnikowin. Drosomycin expression was largely unaffected by loss of the Imd and Toll pathways. Oregon R, not starved or starved (indicated with the line), and ird1, Toll (Tr201Df(3R)roXB3); relish (ref223); imd, spz rm7 (double mutant for Imd and Toll pathways) mutants all subjected to starvation.

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The induction of AMPs under conditions not usually associated with an immune response raises questions as to both the role of AMPs in those processes, and how starvation might impact AMP expression for the immune response. We find if animals are subjected to amino acid starvation, the expression of all the AMPs are lowered compared with that seen with infection alone. The difference is significant ($P < 0.05$) for all but Metchnikowin. In particular, the expression of the AMPs, Attacin, Cecropin, Defensin and Drosocin is dramatically lowered in response to infection (Fig. 5A). In general, the effects of starvation appear to be epistatic over the infection-induced response, as addition of infection shows a similar effect to that of starvation alone. There was no significant difference in the expression of Cecropin, Drosocin, Diptericin and Drosomycin upon infection in the starved animals. For Attacin and Defensin, there was a significant difference upon infection in the starved animals, but the overall level of expression was more similar to that seen instarved uninfected animals than that of well-fed infected animals.

We performed an epistasis experiment to determine where the starvation signal fit relative to the immune signalling pathways (Fig. 5B). We examined whether mutants affecting the Imd pathway (relish and $ird1$), the Toll pathway ($Toll$) or both pathways ($imd$ spätzle double mutant) could still induce Diptericin, Drosomycin and Metchnikowin in response to starvation. Starvation-induced expression of Diptericin and Metchnikowin was dependent on $ird1$ and the Imd pathway. This suggests that $ird1$ and the Imd pathway are downstream of the starvation signal and that the starvation signal either acts through the Imd pathway or works as a co-signal in parallel to the Imd pathway. The starvation induction of Drosomycin appeared to be largely independent of both Imd and Toll pathway function. This suggests that Drosomycin is regulated by a parallel signalling process in starved flies and may be more complex than the Toll pathway-dependent induction of Drosomycin observed in the $ird1$ mutants.

To determine how the starvation-induced expression of AMPs correlated with a response to bacterial infection, we next examined the ability of starved wild-type larvae to survive bacterial challenge (Fig. 6). We find that starved wild-type larvae are not significantly impaired with respect to surviving an E. coli infection as compared with not starved animals ($P = 0.014$), but are significantly impaired in their ability to survive M. luteus infection ($P = 0.014$). The results are surprising based on the known specificity of action of the AMPs on Gram$^-$ and Gram$^+$ bacteria from in vitro and in vivo studies (Lemaître et al., 1997; Tzou et al., 2002; Hoffmann, 2003). The AMPs (Attacin, Cecropin, Drosocin, Diptericin) that are supposed to fight Gram$^+$ bacteria are largely downregulated with starvation, yet the larvae are still resistant to E. coli infection. The AMPs induced by the Toll pathway (Drosomycin, Metchnikowin) are still expressed during starvation, yet the animals are more susceptible to M. luteus infection. This increased susceptibility to Gram$^+$ bacterial infection despite activation of the Toll pathway is similar to what was seen in the $ird1$ mutants. These experiments suggest that a robust AMP expression in response to starvation may be deleterious for survival during a bacterial infection.

**Discussion**

The unexpected discovery that $ird1$, a Vps15 kinase, is important for the innate immune response is a good example of how forward genetic screens can uncover new functions for known genes. This kinase has been primarily
studied for its roles in endocytosis and autophagy (Petiot et al., 2000; Fratti et al., 2001; Futter et al., 2001; Vieira et al., 2001) because the yeast mutant phenotype indicated its importance in cellular trafficking events (Herman et al., 1991; Stack et al., 1995). An examination of the mutant phenotype in a multicellular organism indicates that this kinase also plays a key role in regulating specific NFκB signalling pathways in the *Drosophila* immune response. In *ird1* mutants, the Imd pathway is not activated in response to bacterial infection, whereas the Toll pathway appears to be constitutively activated. This indicates that *ird1* may act at a nexus point for the regulation of both pathways.

The Imd and Toll signalling pathways are often presented as distinct, independent pathways but this is an oversimplified view. Examinations of mutations in the pathways indicate that the two pathways interact. Mutations in the Toll pathway (*spätzle*) show higher levels of Diptericin induction (Vidal et al., 2001); this suggests that the Toll pathway is negatively regulating the Imd pathway and that loss of Toll signalling may result in a compensatory, higher activation of the Imd pathway. *ird1* mutants show a complementary effect with loss of Imd pathway and constitutive activation of the Toll pathway. This may be unique to *ird1*, as other Imd pathway mutants do not show constitutive activation of the Toll pathway. Our results suggest that despite the appearance of constitutive signalling via the Toll pathway in *ird1* mutants, or during starvation, this is actually deleterious for the fly's immune response to bacterial infection. Recent papers also indicate that a constitutive Toll response is harmful and causes flies to be more susceptible to *Drosophila* virus or *Listeria monocytogenes* infection (Gordon et al., 2005; Zambon et al., 2005). Not much is known about how the immune signalling pathways are shut off, but this appears to be as important as activation, for the animal's overall immune competence.

Activation and maintenance of an immune response involves a metabolic cost to the organism. This phenomenon is most apparent when the immune response is always on. In *Arabidopsis*, mutations that constitutively activate systemic acquired resistance (SAR) result in much smaller plants that have reduced fitness (Heidel et al., 2004). In humans, a chronic inflammatory response results in metabolic adaptations to produce acute-phase proteins at the expense of skeletal muscle (Kotler, 2000). Hence, organisms need to find a balance between using available energy resources and mounting an adequate immune response. In *Drosophila*, the fat body serves both as the primary nutrient responsive tissue and as the primary site for AMP production. The requirement for *ird1* for starvation-induced AMP responses and the functional studies of its yeast and mammalian homologues indicate that it is positioned in a nutrient-sensing pathway. Our finding that *ird1* is also necessary to activate the Imd pathway and to keep the Toll pathway in check indicates that *ird1* can affect both the known AMP immune signalling pathways. Having a gene, *ird1*, necessary for both nutrient sensing and immune signalling would provide a means for the organism to quickly integrate signals from these pathways and modulate the strength of its immune response relative to the available energy sources. In the future, it will be important to determine if other components of nutrient sensing pathways can also influence the immune signalling pathways, and whether the mammalian homologue of *ird1*, p150, can affect NFκB immune signalling pathways.

### Experimental procedures

**Drosophila genetics**

Meiotic recombination was used to map *ird1* between the visible markers pink and blister. Mapping relative to deficiencies in the region (DF p-XT103, DF p-XT26, DF p712, DF by10, DF by416, DF by62) indicated that *ird1* was in the 85C1-85D8 interval not uncovered by deficiencies at the time. Male recombination mapping relative to 25 P-elements in the 85C:85D region ([l(3)SO48606, l(3)S129309, l(3)S131505, l(3)S932812, l(3)S07115, l(3)S042008, l(3)S089004, l(3)S049205, l(3)S049906, l(3)S144802, l(3)S093614, l(3)S54507, l(3)S059705, l(3)S138006, EP473, EP501, EP643, EP802, EP3198, EP3260, EP3396, EP3737, KG5749, KG7310, KG7752] further localized the *ird1* map position. All the P-elements were tested for complementation of *ird1* phenotypes. All the lethal P-elements complemented the *ird1* lethality. All the non-lethal P-elements complemented the *ird1* Diptericin-lacZ defect. Plasmid rescue or inverse PCR was used to map the P-element insertion sites to genomic sequence (Huang et al., 2000), if the flanking sequence was not known or posted by the Berkeley *Drosophila* Genome Project. Concurrent with the mapping, the following genes in the region (relish, Nmdmc, Msl85C, CG11033, CG11994, CG18005, CG11990, D1, CG9746) were sequenced in the *ird1* alleles to determine whether they might be candidates for *ird1*. In subsequent genetic screens for mutations that fail to complement the lethality of *ird1*, ethylmethane sulfonate mutagenesis was used to recover the *ird1*3, *ird1*4, *ird1*5 and *ird1*6 alleles and hexamethylphosphoramide mutagenesis was used to recover *ird1*7. For epistasis analyses, double mutants were generated by standard genetic crosses.

**Immune assays**

L3 larvae were placed with filter paper moistened with 1% sucrose in a Sylgard-coated well of a 24-well plate. An overnight *E. coli* culture was resuspended in phosphate-buffered saline (PBS) and injected into the larvae using a microinjection needle and holder attached to a syringe as described previously (Wu et al., 2001). For the *Diptericin-lacZ* assays, 2.5 h after *E. coli* injection, larvae were dissected in 1% glutaraldehyde in PBS and stained with β-galactosidase stain solution (0.2% X-gal in 0.01 M sodium phosphate buffer, pH 7.2, 0.15 M NaCl, 1 mM MgCl2, 0.02% sodium citrate).
3.1 mM K$_2$[Fe$_6$(CN)$_6$], 3.1 mM K$_3$[Fe$_6$(CN)$_6$], 0.3% Triton X-100) at 37°C overnight.

**Amino acid starvation**

The amino acid starvation was essentially performed as described (Scott et al., 2004). Ten to 20 L3 larvae were placed in vials with fresh yeast paste for 24 h to ensure they were well fed before starvation. For starvation, larvae were placed in the Sylgard-coated wells of a 24-well tissue culture dish, with Whatman filter paper soaked with 20% sucrose for 3 h. For the reverse transcription and quantitative PCR, total RNA was made from either well-fed or starved larvae.

**Reverse transcription and quantitative real-time PCR**

RNA was isolated from L3 larvae by homogenizing larvae in STAT-60 buffer according to the manufacturer’s protocol (Isotex Diagnostics). The RNA was digested with RNase-free DNase, then subjected to reverse transcription using Superscript II (Invitrogen) and quantitative real-time PCR using either LUX probes (Invitrogen) on an ABI 5700 following manufacturers’ protocols. Probe sequences are available upon request. AMP gene expression was normalized to Rp49 levels. The different experiments are normalized with the OR$^+$ (infected) expression set at 100% or OR uninfected set at 1. Experiments were performed in triplicate and error bars show standard deviation. One-tail t-tests were used to assess significant differences between samples with $P$-values < 0.05 deemed significant.

**Survival curves**

The larvae were grown on fly food, then either injected immediately with bacteria, or first well fed with excess yeast for 24 h and subsequently starved for 3 h in a 2% sucrose solution. For each experiment 20 L3 larvae were injected using a Pneumatic Picopump (WPI) with either PBS or a 10% washed mid-log culture of either *M. luteus* or *E. coli* in PBS. The experiment was performed in triplicate. Larvae were placed on apple juice agar plates to assess survival for 6 h following injection. Log-rank analyses were used to assess differences in survival curves and $P$-values < 0.05 were deemed significant.

**Generation of *ird1* transgenic rescue flies**

The *ird1* cDNA was reconstructed from three fragments; the 5’ end was reverse transcribed from wild-type RNA in two fragments (0.7 kb, 0.9 kb) and ligated to a cDNA GH20665 (2.4 kb) representing the 3′ end and inserted into pUAST, a transformation vector (Brand and Perrimon, 1993). Transgenic flies expressing the UAS-ird1 were generated using P-element-mediated transformation and the insertions were mapped to chromosomes using standard *Drosophila* crosses. UAS-ird1 insertions on the second chromosome were crossed into lines carrying either the *ird1$^D$* or *ird1$^S$* mutation. The c564 GAL4 driver expressing GAL4 in various immune tissues (fat body and/or lymph glands and other tissues) (Harrison et al., 1995) was crossed into either the *ird1$^D$* or *ird1$^S$* genetic background so that these lines could be crossed to the UAS-ird1; *ird1* lines to generate flies expressing UAS-ird1/GAL4 driver, *ird1$^D$/ird1$^S$.

The [w UAS-ird1 PA10-68] insertion line in the absence of a GAL4 driver resulted in sufficient expression of the transgene for rescue of the *ird1* phenotypes so this was used for the rescue experiments.

**Acknowledgements**

We thank K.V. Anderson for her support during the early stages of this work and for generating four of the *ird1* alleles; M. Nan-dakumar for help in the screen for more *ird1* alleles; R. Scott and T. Neufeld for advice on the starvation protocols; B. Lemaître, D. Ferrandon, BDGP, H. Bellien lab, Exelixis, and the Bloomington and Szeged *Drosophila* Stock Centers for *Drosophila* stocks; the Duke University Model Systems Genomics Unit for transformation services; D. Schneider, S. Wasserman and N. Silverman for helpful discussions; and E. Baehrecke, L. Pick, S. Mount and Wu lab members for helpful comments on the manuscript. The work was supported by start-up funds from UMBI and NIH GM62316.

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Supplementary material

The following supplementary material is available for this article online:

**Fig. S1.** Sequence alignment of *ird1* with homologues from other species. The *ird1/Vps15* gene is highly conserved through evolution and has been identified in most eukaryotic genomes to date. The consensus is shown on the bottom line with the kinase region underlined and the WD40 repeat region underlined with a wavy line. Mutations identified in *ird1* alleles are noted above the aligned sequence. The alignment was performed using MultiAlin and the editing and shading was performed using Boxshade. Some of the *ird1* homologues that show intervening sequences in *ird1* are listed above and are highly homologous regions may be misannotated introns. Ag: *Anopheles gambiae* XP_310476.1, 49% identity; Hs-p150: *Homo sapiens* p150 NP_055417.1, 49%; Rn-p150: *Rattus norvegicus*
Vps15 homologue important for Drosophila immunity

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