Pathogen and host factors are needed to provoke a systemic host response to gastrointestinal infection of Drosophila larvae by Candida albicans

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SUMMARY
Candida albicans systemic dissemination in immunocompromised patients is thought to develop from initial gastrointestinal (GI) colonisation. It is unclear what components of the innate immune system are necessary for preventing C. albicans dissemination from the GI tract, but studies in mice have indicated that both neutropenia and GI mucosal damage are crucial for allowing widespread invasive C. albicans disease. Mouse models, however, provide limited applicability to genome-wide screens for pathogen or host factors – factors that might influence systemic dissemination following GI colonisation. For this reason we developed a Drosophila model to study intestinal infection by Candida. We found that commensal flora aided host survival following GI infection. Candida provoked extensive JNK-mediated death of gut cells and induced antimicrobial peptide expression in the fat body. From the side of the host, nitric oxide and blood cells influenced systemic antimicrobial responses. The secretion of SAP4 and SAP6 (secreted aspartyl proteases) from Candida was also essential for activating systemic Toll-dependent immunity.

INTRODUCTION
Over the past decade the incidence of invasive fungal infection in immunocompromised individuals has dramatically increased, with candidiasis (candidosis) being the most common (Mèan et al., 2008). Although largely due to Candida albicans, a shift towards non-albicans Candida spp such as Candida glabrata, which has reduced susceptibility to commonly used antifungal agents compared with C. albicans, has been recently observed (Mèan et al., 2008). Systemic infection following Candida dissemination is thought to develop from initial gastrointestinal (GI) colonisation (Koh et al., 2008). In cancer patients, C. albicans usually colonises the GI tract with subsequent translocation into extraintestinal organs in the setting of chemotherapy-induced neutropenia and GI mucosal damage (Pasqualotto et al., 2006). In mouse models, three mechanisms promote pathogenic fungal translocation: (1) disruption of the equilibrium in GI flora, thereby permitting intestinal overgrowth of pathogens; (2) increased permeability of the intestinal mucosal barrier; and (3) deficiencies in host defences (Shoham and Levitz, 2005). The majority of those models, however, have employed administration of a chemotherapeutic agent (e.g. cyclophosphamide) followed by the subsequent intravenous administration of C. albicans (Andes et al., 2003; Coligan, 2001). A mouse model that first establishes GI colonisation followed by dissemination after disruption of various components of innate immunity was only recently reported (Koh et al., 2008).

Nevertheless, murine models are labour and cost intensive, and as a consequence offer low statistical resolution. Moreover, they provide limited applicability to genome-wide screens for pathogen or host factors; these factors might influence systemic dissemination following GI colonisation. For this reason, we developed a Drosophila model to study intestinal infection by Candida. The effects of Candida GI infection in Drosophila were previously unexplored. Until now, the Drosophila-Candida interaction models paralleled the mice intravenous model, with pathogen administration by injection into the body cavity (Chamilos et al., 2006; Alarco et al., 2004). Studies on bacterial-modulated gut responses in Drosophila (for a review, see Lee, 2009) have provided a global view on the host genes that respond to non-pathogenic (Bouchon et al., 2009a) and/or pathogenic (Cronin et al., 2009) bacteria. In relation to non-invasive non-pathogenic Erwinia carotovora carotovora infection, the host genes identified were related to stress response, cell growth, wound repair and development (Bouchon et al., 2009a). Significantly, several of these processes are likely to be dependent on an intact dual-oxidase (DUOX)-dependent reactive oxygen species (ROS) immune response (Ha et al., 2005). Multiple genes were also shown to be involved in defence against the lethal pathogen Serratia marcescens, namely genes involved in proteolysis, transport, stress response, immune response, growth, wound repair and cell death. One important aspect of host responses to GI infection in adult flies is the repair mechanism of the gut, which loses enterocytes (ECs) as ‘co-lateral’ damage during pathogen killing, presumably through the release of ROS. Generation of ROS, however, also stimulates intestinal stem cell (ISC) activation: a reduction in ROS levels results in a reduction of microbe-induced ISC proliferation (Bouchon et al., 2009b). In the course of gut renewal (owing to physiological EC turnover, epithelial injury or microbial infection), ISCs proliferate...
and give rise to enteroblasts, which will in turn differentiate into ECs and secretory enteronecrotic cells. Multiple signalling pathways (JAK/STAT, Notch and JNK) are involved in ISC activation, each at a different level of the process (for a review, see Lee, 2009). Flies lacking the ability to control normal gut flora equilibrium owing to the absence of innate immune signalling in the gut [namely, absence of the immune deficiency (imd) pathway, meaning that antimicrobial peptide (AMP) gene expression is compromised] show an overproliferation of ISCs and abnormal gut morphology. This is abolished if flies are raised in germ-free conditions (Bouchon et al., 2009a).

An additional signal during GI infection in Drosophila larvae is nitric oxide (NO). NO is a diffusible, reactive and membrane-permeable small molecule synthesised from L-arginine by the enzyme nitric oxide synthase (NOS) (Knowles and Moncada, 1994; Nathan and Xie, 1994). At low concentrations in vertebrates, NO plays a signalling role, regulating innate and adaptive immune responses both positively and negatively (for a review, see Guzik et al., 2003). This is paralleled in insects: NO has also been shown to induce cellular and humoral immune responses in Drosophila (Nappi et al., 2000; Foley and O’Farrell, 2003), the silkworm Bombyx mori (Imamura et al., 2002) and the lepidopteran Manduca sexta (Eleftherianos et al., 2009). In Drosophila larvae, NO signalling is important for the induction of AMPs in the fat body following oral Gram-negative bacterial infection (Foley and O’Farrell, 2003). NO function is mediated by haemocytes and more specifically by Calcineurin (CanA1) expressed within these cells (Dijkers and O’Farrell, 2007). CanA1 RNA interference (RNAi) in haemocytes was sufficient to block immune induction of AMPs in the fat body, but CanA1 RNAi in the fat body was not. It was thus postulated that CanA1 provides an additional input into AMP systemic activation, and functions in haemocytes to promote a tissue-to-tissue signalling cascade required for a robust immune response.

In addition to GI responses, Drosophila mounts an efficient systemic innate immune response against disseminated infection, the hallmark of which is the production by the fat body and release into the blood of a battery of potent AMPs (for reviews, see Lemaitre and Hoffmann, 2007; Wang and Ligoxygakis, 2006). This antimicrobial activity can persist for several days and is specific according to the broad category of the invading pathogen. The signalling pathways that control the production of AMPs are activated by the interaction of host pattern recognition receptors (PRRs) with molecules on the surface of fungi and bacteria, or by secreted virulence factors (see below). PRR families include the peptidoglycan recognition proteins (PGRPs) and the glucan binding proteins (GNBPs). Fungal recognition in particular is attributable to the detection of fungal cell wall by GNBP3, and of secreted fungal proteases via the activation of the protease Persephone (Psh) (Gottar et al., 2006). Pathogen detection by either mechanism triggers proteolytic cascades, which converge on the proteases Grass and Spätzle-processing enzyme (SPE) (Jang et al., 2006; El Chamy et al., 2008). The latter is responsible for the proteolytic activation of the cytokine Spätzle (Spz), which in turn acts as a ligand for the transmembrane Toll receptor (Weber et al., 2003). This activates the Toll-signalling pathway, which culminates in the translocation of the NFκB-like transcription factor Dif to the nucleus (Rutschmann et al., 2000a). There, Dif activates, among others, the antifungal peptide gene Drosomycin (Drs), which is regularly used as a read-out for Toll-pathway activity following immune challenge.

We have previously shown that clinical isolates of C. albicans exhibit the same virulence (measured as median time of host survival) in both flies and mice when injected to activate disseminated candidiasis [see accompanying paper in this issue (Glittenberg et al., 2011)]. This result and the extensive conservation between Drosophila and mammalian innate immune responses led us to explore the fruit fly model with the view to pinpoint genes from both Candida and Drosophila that are involved in activating systemic infection following GI colonisation. We first established GI infection in Drosophila larvae and followed the consequences of infection on gut physiology, larvae survival and development to adult. GI infection with C. albicans provoked a systemic response that was GNBP3 independent but Psh-Toll dependent and required the presence of haemocytes. NO modulated this response: specific knockdown of NOS in the gut significantly reduced the levels of Drs in the fat body following GI infection. In addition, proteases secreted by Candida were necessary to provoke systemic immunity.

RESULTS C. albicans GI infection induces systemic immune activation in Drosophila larvae

To establish survival rates to adulthood of Drosophila larvae that were fed C. albicans, we set up infections of early third instar larvae with food in the presence or absence (control) of Candida (for larval staging and infection procedure see Methods). We analysed wild-type larvae as well as larvae that were simultaneously deficient for both the Toll and Imd pathways (dif, key) (Rutschmann et al., 2002). Dif is the NFκB transactivator downstream of the Toll receptor-adaptor complex (Rutschmann et al., 2000a) (see Introduction), whereas Kenny (Key) is the Drosophila IKK homologue essential for Imd signal transmission (Rutschmann et al., 2000b). The number of larvae that developed into adult flies was comparable between wild type and mutants (Fig. 1A). On average, 90% of wild-type and 83% of dif, key larvae fed on normal food survived to adulthood. Comparably, 83% of wild-type larvae and 70% of dif, key larvae that were fed normal food containing C. albicans developed into adult flies (Fig. 1A). However, the result was totally different when the parental generation was grown in germ-free (GF) conditions (see Methods; GF larvae). In this case, the number of wild-type larvae developing to adults was significantly lower than when the parental strain was not grown in GF conditions (58%) and that of dif, key mutants was dramatically reduced to 22% (Fig. 1A). These data indicated that indigenous bacterial flora acted antagonistically towards Candida (and/or assisted host survival), because as soon as they were removed the effect of Candida on host survival was much more pronounced. This correlates with data from human patients with vulvovaginal candidiasis, in which disease manifestations occur when the balance of normal flora is disrupted (Sobel, 1992). In addition, Candida infection of GF larvae compromised somewhat the survival of spz or imd mutant larvae (Fig. 1A). However, this was not the case in normal food (data not shown). This indicates the importance of the normal flora in reducing Candida colonisation and underlines the interplay between the immune system and resident microbes during colonisation by an invading pathogen. Nevertheless, spz did play a
role both in survival and the mounting of a systemic response (see below).

In GF conditions, JNK-mediated cell death was the cause of death in wild-type larvae. We expressed the JNK target (and at the same time suppressor of the pathway) puckered (puc) in larval ECs (through NP1-GAL4, tub-GAL80ts) (Fig. 1A). GAL80ts (McGuire et al., 2003) was used to avoid any effects that overexpression of puc might have had in the gut earlier in development. At the permissive temperature (17°C), GAL80 is ON, keeping GAL4 OFF, whereas at restrictive temperature (25-29°C), GAL80 is OFF and GAL4 ON. When puc was expressed in ECs (29°C), survival to adulthood in wild-type larvae following Candida GI infection was restored to 78.2% (with s.d. 7.61; Fig. 1A), which is comparable with the survival of non-GF-reared wild-type flies (83%). Unfortunately the converse experiment, in which GAL80 was active (17°C) and therefore puc expression was suppressed, was not meaningful because Candida growth at 17°C was limited and it was not able to induce a systemic response in Drs-GFP larvae (our unpublished observations). Nevertheless, the effect of puc on survival was reminiscent of recently published data for Pseudomonas aeruginosa (Apidianakis et al., 2009). Inactivation of JNK therefore rescued cell death following Candida GI infection.

C. albicans GI infection induced systemic activation of Drs in the fat body in flies raised with both normal or GF food (data not shown; see below). Using a Drs-GFP transgene (Ferrandon et al., 1998), we followed the time course of Drs activation in infected larvae. On average, 80% of larvae had a full fat body induction at 5 hours post-infection (not shown). Fig. 1B summarises experiments conducted in normal food. As shown, systemic Drs induction was dependent on Toll signalling: larvae mutant for spz showed dramatically reduced Drs expression. By contrast, imd was dispensable for Drs expression in the fat body. Both results were to be expected because, in fruit flies, it is the Toll pathway that controls systemic responses to fungal infection (see Introduction). Moreover, Drs expression required the presence of haemocytes. GI-infected larvae devoid of blood cells exhibited on average a tenfold induction of systemic Drs, in comparison with the 28-fold activation seen in wild-type larvae (Fig. 1B). Haemocyte-depleted larvae had a severely reduced number of haemocytes, owing to the expression of the pro-apoptotic genes head involution defective (hid) and reaper (rpr) in a haemocyte-specific manner (see Shia et al., 2009). It is interesting to note here that, regardless of the systemic activation of Drs, Candida was contained in the larval gut. Using Act-GFP-Candida (GFP expressed via an Actin promoter) (Barelle et al., 2004), we never observed Candida outside of the gut (data not shown). In addition, after carefully bleeding infected larvae on microscopy slides and plating the haemolymph (see Methods), no Candida GFP-containing colonies grew (our unpublished observations). However, if Candida was contained in the gut, what was the physiological significance of the systemic response and why do GF larvae die during their development to adulthood? We speculate that, during pupariation, the gut empties and this might potentially be detrimental to the host. Indeed, using Act-GFP-Candida, one could observe this following puparium formation (data not shown). In addition, a considerable proportion of the spz larvae and, more importantly, of the nos-RNAi; spz double-mutant larvae (see below) raised in GF conditions were not able to reach adulthood when infected gastrointestinaly (our unpublished observations).

Fig. 1. Candida GI infection has adverse consequences in Drosophila larvae survival to adulthood. (A) Survival of Drosophila larvae following GI infection in germ-free (GF) and normal food conditions. Values in the graph are mean values ± s.d. of three independent experiments. Values are % of larvae surviving to adulthood. Values indicated by identical symbols are not significantly different (P > 0.05) from each other. All other differences are statistically significant (P < 0.05). In particular, P = 0.00017 between UAS-puc and dif, key GF + Candida and P = 0.026 between UAS-puc and wild-type (WT) GF + Candida. (B) The Toll pathway: haemocytes but not Imd was needed for activation of systemic Drs following GI infection. Drs induction was measured by quantitative real-time PCR in triplicates for each biological sample. Each value presented in the graph is a mean value from three independent experiments. Values indicated by identical symbols were not significantly different (P > 0.05) from each other. All other differences were statistically significant (P < 0.05). WT unchallenged was used as a base line (set at 1) to compare fold induction.
Candida GI infection triggers extensive cell death in the gut epithelium

Ingestion of Candida resulted in extensive cell death in a relatively short time. Using a STAT–destabilised-GFP Drosophila strain (STAT-GFP\textsubscript{destab}) (Bach et al., 2007) in which GFP is only expressed in ISCs (Bouchon et al., 2009a; Bouchon et al., 2009b), we followed JAK-STAT pathway activity during infection of larvae grown in normal food. As pathogenesis progressed, we recorded fewer GFP cells (at 4 and 9 hours post-infection) accompanied by a progressive reduction of epithelial lining (Fig. 2A1-A3). Disappearance of STAT-GFP\textsubscript{destab} was not caused by cells switching off the JAK-STAT pathway but rather by extensive cell death (Fig. 2B, compare with the uninfected control in 2C), something that would correlate with the observed loss of epithelial tissue. Cell death in the gut epithelium owing to bacterial infection or stress has been reported by numerous recent studies (Apidianakis et al., 2009; Bouchon et al., 2009a; Bouchon et al., 2009b; Jiang et al., 2009) (for a review, see Lee, 2009).

Candida proteinases activate systemic immunity

We next wanted to pinpoint the pathogen features that activate systemic responses following GI infection. The most recent working model for systemic triggering of the Toll pathway in Drosophila defines two possible routes of activation. One is through pathogen-secreted proteinases that activate Toll via Psh, whereas the other is through cell wall sensing via GNBP3 (Gottar et al., 2006; El Chamy et al., 2008). In our experiments (normal food), 96% of larvae that were infected gastrointestinally with Candida exhibited systemic activation of their immune response as judged by expression of a Drs-GFP reporter (Fig. 3A, food + unwashed Candida column). These were scored ‘positive’ only if they expressed Drs-GFP in the whole of their fat body (Fig. 3B). By contrast, only 5% of Drs-GFP larvae that were fed C. albicans but not infected had such a systemic activation. We infected larvae with Candida washed extensively with PBS to remove discarded or recycled cell wall fragments, as well as with heat-killed Candida (washed and unwashed) to denature any secreted proteinases and stop Candida cells from producing more. As seen in Fig. 3A, only infection with heat-killed Candida significantly reduced (from 100% to 33%) the percentage of larvae that showed systemic induction of Drs-GFP. Washing did not have an effect on systemic immune activation: 95% of infected larvae still expressed Drs-GFP in the whole of their fat body tissue. From the host side, larvae that were mutant for psh were unable to mount a proper response when gastrointestinally infected with live Candida, resulting in just 32% of larvae showing systemic Drs-GFP expression (Fig. 3C). By contrast, 92% of gnbp3 mutant larvae expressed Drs following the same immune challenge (Fig. 3C).

These results indicated that secreted proteinases could be involved in the activation of systemic immunity. C. albicans secretes only one class of proteinases, so-called SAPs (for secreted aspartyl proteinases).
proteinases), which has ten members (for a review, see Naglik et al., 2004). These proteins have been associated with Candida virulence in mice and human reconstituted vaginal tissue in both the yeast (SAPs 1-3) and hyphal stages (SAPs 4-6). In a mouse model of GI infection, SAP4 and SAP6 were constitutively expressed, with SAP2, SAP3 and SAP5 mRNA only occasionally being detected (Cassone et al., 2002). Similar data, again from mouse models, indicate that SAP genes are upregulated in biofilms, a predominant feature of mucosal candidiasis (García-Sanchez et al., 2004). We infected larvae with a number of available mutant strains with knockouts of individual SAPs or combinations (see Fig. 3D and Methods). These mutants were generated in the wild-type SC5314 background that we used as our wild-type reference strain (Naglik et al., 2004). Colony-forming unit (CFU) measurements indicated that, following 5 hours of infection, the amounts of living mutant vs wild-type Candida in the host were comparable (supplementary material Fig. S1). In addition, cell death and reduction of epithelial lining in the host gut was similar between infection with SAP mutants or wild-type Candida (data not shown). Our results indicated that the absence of SAP4 and SAP6 considerably reduced systemic activation of Drs to an average threefold induction from an average of 13-fold induction when larvae were challenged with wild-type Candida (Fig. 3D). This was, in turn, reminiscent of mouse models (see Discussion).

If GNBP3 participated in the activation of systemic infection following ingestion then heat-killed Candida would activate the system less in gnbp3 mutants than in wild-type larvae. This is due to the following: in wild-type larvae, heat-killed Candida would not be able to induce the Psh branch that leads to Toll signalling. In gnbp3 mutants, however, heat-killed Candida would not be able to trigger Toll by any of the two branches (protease or cell wall recognition). Interestingly, GI infection of gnbp3 mutants with heat-killed Candida activated Drs at the same reduced levels as in wild-type larvae, as measured by quantitative real-time PCR (qPCR; Fig. 3C). Finally, double psh; gnbp3 mutant larvae exhibited a similar reduction of Drs as psh single mutants following GI infection with live Candida (Fig. 3C). In addition, the Candida cell wall mutant PMRI, which has defects in both N- and O-linked mannosylation (Bates et al., 2005), activated Drs to the same extent as did wild-type Candida (Fig. 3C). One possibility not excluded by these experiments is that a fungal cell wall recognition receptor other than GNBP3 might be at play in larvae. Nonetheless, Drs expression was never completely abolished in any of the single or double mutant combinations.
This result prompted us to look for additional potential inductive signals emanating from the gut.

**NO acts as a host signal emanating from the gut and is crucial for systemic induction of Drs**

Because NO has been documented to modulate systemic AMP expression following GI bacterial infection in Drosophila larvae (see Introduction), we monitored the occurrence of site-specific induction of NOS by northern blot analysis after ingestion of Candida. We carefully separated constituent tissues from haemocytes (the latter by bleeding larvae on microscopy slides) and then dissected larvae into four parts: gut, fat body, haemolymph and carcass (including salivary glands and Malpighian tubules). To pinpoint any fat body cross-contamination in any of the tissues, along with NOS we monitored expression of larval serum protein 2 (lsp2), which is exclusively and very strongly expressed in the fat body (Powell et al., 1984; Aguila et al., 2007). As seen in Fig. 4A, we found that, following infection, NOS is predominately activated in the gut, as has been found in other insects (Hao et al., 2003; Eleftherianos et al., 2009). We then used a UAS construct expressing a double-stranded RNA against NOS (UAS-RNAi-NOSIR; see Methods) for tissue-specific knockdown. We combined this with NP1-GAL4 and tub-GAL80ts (McGuire et al., 2003) to avoid any developmental effects that NOS silencing might have had in larvae (Regulski et al., 2004). Following these experiments, another group published a study that showed that NOS was not essential for development in fruit flies (Yakubovich et al., 2010).

Fig. 4B shows that the construct driven by the gut-specific NP1-GAL4 driver was successful in silencing NOS at the mRNA level at the restrictive temperature (GAL4 ON, GAL80 OFF; see Methods).

In addition, UAS-RNAi-NOSIR expressed via Tubulin-GAL4 was efficient in knocking down NOS protein in the central nervous system (supplementary material Fig. S2), the larval tissue where NOS was found to be expressed the most during development (Kuzin et al., 1996). Knockdown of NOS in the gut through NP1-GAL4 had a significant effect on Drs expression in the fat body, reducing it to 32% of wild-type levels (Fig. 4B). NOS knockdown in haemocytes (hemolectin-GAL4) or the fat body (lsp2-GAL4) did not have an effect on Drs expression (data not shown). Results that were comparable to the NOS gut knockdown were obtained when the NOS inhibitor N-omega-nitro-L-arginine-methylester (L-NAME)
was added to the food, but not when its inactive D-enantiomer D-NAME was added, as revealed by qPCR in whole larvae (Fig. 4C). There again, Drs expression was significantly reduced from an average of 12-fold induction in gastrointestinal infected larvae that were not treated pharmacologically to an average of 4.5-fold induction in larvae raised in L-NAME-containing food.

**Host and pathogen factors contribute to activation of systemic immunity following GI infection**

The results presented so far indicated two aspects of GI Candida infection that quantitatively modulated activation of systemic immunity in the fat body, namely pathogen-secreted proteinases (SAPs) and the synthesis and release of NO by the host. It has been previously shown that the signalling influence of NO requires the presence of haemocytes (Foley and O’Farrell, 2003; Dijkers and O’Farrell, 2007). This correlates with our observation that larvae devoid of haemocytes were unable to mount a response (see Fig. 1B). This systemic response was also dependent on the Toll pathway, because lack of the ligand of the pathway (spz) (or of the upstream protease psh) results in a significant reduction of Drs expression (Fig. 1B). It is tempting to speculate that SAPs activate the Toll pathway via psh, as per the working model for activation by pathogen-derived ‘danger signals’ (see El Chamy et al., 2008), whereas NO is a host signal used as an independent input in modulating systemic activation. If this were the case then loss of either of these individually (the NO or Toll signals) would not abrogate Drs expression but merely reduce its induction, which is what we observe. We were therefore interested to see whether larvae deficient for both these signals would have no induction of Drs, as predicted by the above model. Indeed UAS-NOSRNAi/Y; NP1-GAL4, GAL80ts; spzrm7 larvae had a dramatically reduced induction of Drs that was on average 10% of the normal Drs activation of wild-type larvae (Fig. 5A). Moreover, in GF conditions, survival of UAS-NOSRNAi/Y; NP1-GAL4, GAL80ts; spzrm7 larvae to adulthood following GI Candida infection was severely compromised, with an average of 4% of larvae becoming adults, compared with 93% of UAS-NOSRNAi/Y; spzrm7/+ larvae surviving to adulthood (Fig. 5B). It is interesting to note here that GFP-Candida was not seen escaping from the gut into the haemolymph in either NOS-RNAi or NOS-RNAi; spz larvae, although we did detect Candida crossing the border between the gut and the Malpighian tubules in both cases (data not shown).

![Fig. 5. Pathogen and host factors contribute to systemic activation of Drs.](image)

**A** Activation of Drs in a background in which both NO and the Toll pathway are compromised (UAS-RNAi-NOSRNAi/Y; NP1-GAL4, GAL80ts; spzrm7), as seen using qPCR (mean values from three independent experiments ± s.d.). Ratios of unchallenged UAS-RNAi-NOSRNAi/Y; spzrm7/+ controls were anchored in 1 to indicate fold induction. Values indicated by identical symbols were not significantly different (P>0.05) from each other. All other differences were statistically significant (P<0.05). **B** Additionally, survival to adulthood of UAS-RNAi-NOSRNAi/Y; NP1-GAL4, GAL80ts; spzrm7/+ larvae grown in GF conditions and then infected with Candida was dramatically reduced compared with controls. P=0.00012. **C** A model depicts schematically the events leading to fat body response following Candida GI infection (see Discussion).
DISCUSSION

Taken together, our results show that there are two signals synergising to activate systemic Drs expression (Fig. 5C). It is the combination of both host NO signalling and pathogen-derived ‘danger signals’ that is important for the synergistic induction of Drs in the fat body, with both processes independently converging to give this response. Pathogen signals that activated Toll-dependent systemic immunity through Psh were the proteases SAP4 and SAP6. It is relevant to note here that these two SAPs are believed to have the same substrate range (Nglik et al., 2004). Moreover, they were constitutively expressed in a mouse model of GI infection (Cassone et al., 2002) and were found to have a role in systemic infections in mice (in contrast to SAPs 1-3) (Kretschmar et al., 1999; Felk et al., 2002). We believe, however, that there should be additional pathogen factors that influence activation of systemic immunity because Drs was not completely abolished in the single mutants, although that might have been the case in a SAP4, SAP6 double mutant. The set up of this host-pathogen interaction system should be relevant to explore other pathogen factors contributing to the modulation of systemic immunity (see below).

Results from bacterial infection studies have established that NO signalling is mediated by CanA1, which is required in haemocytes, and members of the Toll-independent Imd pathway upstream of the NFκB homologue Relish (Foley and O’Farrell, 2003; Dijkers and O’Farrell, 2007). Our data confirm that haemocytes are required to transmit the host signal to the fat body, because genetic ablation of blood cells significantly reduced Drs expression. This is the first time that Drs expression has been shown to be partly Toll independent. Although this remains to be established, we anticipate that Imd would again be the regulator of Drs induction, because the NO reaction is by no means Candida specific.

Candida is an opportunistic pathogen that is a commensal for a large part of the human population. When under immunosuppression, however, carriers can be infected with their own resident Candida, which thus turns into a systemic threat. Nevertheless, not a lot is known about which pathogen factors drive forward such systemic infections. As a model, Drosophila has already been widely used as an understudy for human gut pathogenesis (for a review, see Apidianakis and Rahmet, 2011). In this paper, we developed and exploited a fruit fly GI infection model to study C. albicans and host factors required for disseminated infection. This model could be further exploited to harness the power of Drosophila as a high-throughput system. By using the vast array of knockout mutants available for Candida spp, one would be able to make whole library screening using Drs-GFP larvae and pinpoint pathogen factors that modulate systemic activation of Drs in the fat body. Conversely, screening using the human homologues of the Drosophila genome in gut-specific knockdown would identify host genes implicated in pathogenesis and/or involved in defence against the pathogen that are relevant to mammalian models.

METHODS

C. albicans strains and procedures

For preparation of inocula for all experiments, Candida was cultured as yeast cells in 5-ml volumes of NGY medium (0.1% Neopeptone, 0.4% glucose, 0.1% yeast extract). Working subcultures on Sabouraud agar were maintained at 4°C for a maximum of 14 days. The SC5314 strain was used as the wild-type C. albicans strain. The PACT1-GFP strain (GFP-Candida) (Barelle et al., 2004) was used to verify infection and ascertain whether the pathogen was contained in the gut. SAP mutants C66, C68, C72, C73, C74 and C109 (gift from Neil A. R. Gow, University of Aberdeen, Scotland) were used for studies on the importance of SAPs for pathogen recognition. The mutant strains were C66 (sap1), C68 (sap2), C72 (sap4), C73 (sap5), C74 (sap6), C109 (sap5, sap6) [see Hube et al. (Hube et al., 1997) for C66 and C68, and Sanglard et al. (Sanglard et al., 1997) for C72, C73, C74 and C109]. For heat inactivation, C. albicans was incubated at 90°C for 3 hours and then allowed to cool before feeding it to the larvae. To remove cell wall components, Candida was washed with 1× PBS ten times before feeding to the larvae. When doing these in combination, Candida was heated and then washed.

Drosophila stocks and procedures

All stocks were isogenised before this study was initiated. We used Oregon R flies as the wild-type strain. Additionally, we used dif key (Rutschman et al., 2002); spe"+" (Morisato and Anderson, 1994); imd" (Lemaître et al., 1995); UAS-puc (Martin-Blanco et al., 1998); NP1-GAL4 (ECs); esg-GAL4 (ISCs and enteroblasts) (Michelli and Perrimon, 2006); Drs-GFP (Ferrandon et al., 1998); Stat92E-GFP destabilised reporter (Bach et al., 2007); UAS-hid, UASspr; hml-GAL4 (Shia et al., 2009); tub-GAL80" (McGuire et al., 2003); and UAS-NOS-RNAi (this study). All stocks were maintained at 25°C in standard food except UAS-hid, UASspr; hml-GAL4 flies, which were maintained at 18°C. GF parental stocks were generated by bleaching embryos and cultivating them on autoclaved polenta-agar medium. Emerging flies were maintained (or crossed) on autoclaved standard medium. During the NOS knockdown or puc overexpression, the activity of the GAL4 system was controlled by placing progeny either at restrictive temperature (29°C; GAL80 OFF, GAL4 system ON) or at permissive temperature (17°C; GAL80 ON, GAL4 system OFF). It has been reported that full GAL4 activity is not achieved until 6 hours after temperature shift owing to the perdurance of GAL80 (McGuire et al., 2003). In the relevant experiments we infected larvae 6 hours after the temperature shift.

To determine the proportion of NOS induction in different tissues, 40 larvae of each strain (wild type, NP1-GAL4 or NOS-RNAi; NP1-GAL4) were recovered 6 hours post-infection and dissected in 50 μl of ice-cold PBS using a fine pair of forceps on ice. The forceps were thoroughly cleaned after each dissection with a clean paper towel and water to avoid tissue cross-contamination. Briefly, each larva was decapitated then carefully rolled inside out on the tip of a forceps. The fat body was carefully dissected and placed in a microfuge tube on ice. To prevent disintegration of the fat body, larvae were dissected in sets of five as quickly as possible and frozen at −80°C. Hemolymph with blood cells (drained and bled into cold PBS during dissection) was pooled together, as were the gut and the carcass (containing the cuticle, brain, trachea, salivary glands and Malpighian tubules that were separated from the gut) before RNA extraction using Trizol (Invitrogen) according to the manufacturer’s instructions. In each case (whole larvae, fat body, carcass, haemolymph), 20 μg of total RNA were separated on a formaldehyde gel for northern blot analysis as previously described (Ligoxygakis et al., 2002). Northern blot experiments presented in this study were independently repeated at least three times.
times. Graphs represent the mean and s.d. of % ratios, where the wild-type infected strains were docked as 100%. Experiments with L-NAME and D-NAME were performed as described (Brown et al., 2009).

Gut dissections were performed in cold PBS. Dissected guts were then put into the fixative for 20 minutes before staining with 4′,6-diamidino-2-phenylindole (DAPI) and (when appropriate) with Sytox green in PBS at room temperature for 20 minutes. DAPI and Sytox green were both diluted to 1/1000 in 1× PBS before adding the guts for staining. DAPI and Sytox green are both nuclear stains. However, DAPI is able to cross the membranes of cells, whereas Sytox green cannot. This means that Sytox green can only stain DNA of cells with compromised membranes like the ones undergoing apoptosis, whereas DAPI will stain live and dead cells. Four successive washes with PBS were performed to remove excess stain before putting the dissected guts on slides in VECTASHIELD mounting medium (Vector Laboratories). Images of dissected guts were taken using a Zeiss Axioplan 2 microscope with AxioCam.

**Infection protocol**

A concentrated culture of *Candida* was mixed with an equal volume of the usual food for the larvae and this mixture was spread thinly onto the surface in the centre of an apple juice agar plate. As a control, plates would be prepared with only the food spread thinly on a plate. 20 or 30 late second instar or early third instar larvae were then placed onto each plate before sealing with tape. Larval staging was as described (Demerec, 1950). Often, larvae were found stuck to the tape around the plate. Such larvae were omitted from counts or any other measurement. The larvae would feed on the *Candida*/food mix for 6 hours before being removed and washed in a small amount of water prior to putting back in fresh food vials for survival count to adulthood, or observation of *Drs-GFP* activation, or dissection for gut staining, or freezing for either northern or qPCR. All infection experiments were done at 30°C.

Supplementary material Fig. S3 documents the infection process using *GFP-Candida*.

**Microscopy**

Larvae were visualised on a GFP stereo dissecting microscope (Leica MZFIII, UK), and images captured using KyLink software (v2.0, Japan) and the JVC KY-F75U 3CCD monochrome camera (JVC, Japan). Images were colour corrected and assembled using Adobe CS2 (Adobe, USA Mac OSX).

**Systemic response activation analysis by qPCR**

Total RNA was isolated from the larvae using TRIzol reagent (Invitrogen). 5 μg of the isolated RNA was treated with DNase I (recombinant, RNase free; Roche Applied Science) to remove genomic contaminants (Schmittgen and Livak, 2008). 100 ng of the purified RNA from each sample was reverse transcribed to produce cDNA with the AffinityScript Multiple Temperature cDNA Synthesis Kit (Agilent Technologies) using random primers in a total reaction volume of 20 μl. 2 μl of the processed cDNA synthesis reaction mixture (10 ng of total RNA) were used as templates for qPCR, which was carried out with the Brilliant II SYBR Green QPCR Master Mix (Agilent Technologies) according to the manufacturer’s instructions, using relevant primers to quantify *Drs*, *NOS*, *lsp2* and *Rp49* mRNA. Amplification was performed with 40 cycles of 30 seconds at 95°C and 1 minute at 60°C after an initial 10 minutes at 95°C to activate the DNA polymerase. Each primer was at a concentration of 400 nM in a total reaction volume of 25 μl. The reactions were carried out in separate 0.1-ml reaction tubes for each set of primers in triplicate in a QIAGEN Rotor-Gene Q real-time PCR cycler and preliminary data analysis was carried out with the accompanying software (Rotor-Gene Q v1.7.94 for Windows). Gene expression of *Drsp*, *NOS* and *lsp2* relative to the expression of *Rp49* was determined using the 2−ΔΔCt method (Schmittgen and Livak, 2008). Relative DCt*Drsp−DCtRp49* ratios of unchallenged wild-type controls were anchored in 1 to indicate fold induction. Graphs represent the mean and s.d. of relative ratios detected in three biological repetitions of a pool of ten females.

**Construction of UAS-NOS**

A 696-bp fragment of part of exon 16 plus the complete following intron was amplified and cloned into pGEM T EASY (Promega) using primers 5′-CTCTGATTCGCCGCCTGCACTGCTGGTT-3′ *EcoRI* (CTCGGGCGCCTGGATGACTGACGGGCGAT-3′ NotI). A 457-bp fragment of exon 16 was amplified with primers 5′-CTCCTGAGGCGCCGTCAGCCTGCCTGT-3′ *HindIII* (CTCGGAATTCGCCCTGCACTGCTGGTT-3′ XhoI). Both products were cloned into pGEM T EASY. Sequences not derived from *Drosophila* NOS are underlined. Each fragment was then sub-cloned from pGEM T EASY as an *EcoRI-NotI* fragment of exon 16 plus intron and NotI-XhoI fragment of exon 16 in reverse orientation into pUAST (Brand and Perrimon, 1993). The completed construct (UAS-NOS) was transformed essentially following the method of Spradling and Rubin (Spradling and Rubin, 1982): 0.4 mg/ml *pUAST* construct and 0.1 mg/ml pdelta2-3 in injection buffer (5 mM KCl, 0.1 mM NaPO4, pH 7.8) was injected into *yw* larva <30 minutes old. No off-targets of this RNAi construct were detected using a web-based search tool (http://www.dlfkz.de/signaling/e-rna3//). 4- to 7-day-old adults flies were used in all experiments.

Western blot for the detection of *Drosophila* NOS expression

Primary antibody against NOS was used in 1/3000 dilution (Regulski et al., 2004) with the subsequent use of an anti-rabbit-HRP (horse radish peroxidase)-conjugated secondary antibody in 1/2000 dilution (Chemicon International). In order to quantitate equal protein loading of the same samples used for anti-NOS antibody detection, anti-Actin (Sigma A-2066 Rabbit Lot 083K4834) was used between 1/2000-1/2500. The PVDF membrane was blocked for 1 hour in 5% skimmed milk powder with 3% BSA in 1× Tris-buffered saline and Tween-20 (TBST). The primary antibody was diluted in blocking solution and incubated at 4°C overnight, on a rocker. The membrane was subsequently rinsed five times with 1× TBST for 5 minutes at room temperature; the secondary antibody was added diluted in blocking solution and incubated at room temperature. The membrane was rinsed five times with 1× TBST for 5 minutes each. 1-2 ml of ECL solution (Amersham Biosciences) was incubated for 5 minutes at room temperature. The membrane was exposed to photographic film and developed, using Amersham Biosciences Hypercassette RPN 13642, Hyperfilm RPN3103K and Konica SRX-101A medical film processor.
Clinical issue

*Candida albicans* is a common diploid, polymorphic fungal organism that can colonise mucosal surfaces – frequently beginning in the gastrointestinal (GI) tract – in healthy human adults; one study reports benign, commensal occurrences of *Candida* spp in over 50% of the population. However, impairment of host defence mechanisms due to underlying immune deficiencies, such as infection with HIV, prolonged chemotherapy, major surgery, immunosuppressive treatments or extremes of antibiotic use, can predispose individuals to infections ranging from mucocutaneous forms of candidiasis to systemic infections such as candidaemia and invasive candidiasis. *Candida* has thus risen to be the fourth most common form of bloodstream infection in the USA over the past three decades. Such systemic infections exhibit an associated mortality rate estimated at >30% in the USA, at >25% in the UK and at >35% in Europe; of the reported deaths, ~30-50% are directly attributable to the incidence of candidemia. *C. albicans* is responsible for up to 80% of life-threatening disseminated *Candida* infections and is the most common fungal pathogen in humans. However, little is known about the host and pathogen factors that contribute to systemic immunity following a *Candida* GI infection.

Results

This paper demonstrates that the fruit fly *Drosophila melanogaster* is an appropriate host model to explore the basic biology of host-*Candida* interaction following GI infection. The authors show that infection of fly larvae by giving them *Candida*-spiked food induces cell death in the gut that involves both enterocytes and stem cells. This infection also increases larval mortality, but only when parental flies are reared in germ-free conditions; as in humans, the normal flora of the gut (which is absent in germ-free larvae) protects against *Candida* infection. Inactivation of the JNK signalling pathway in the gut of germ-free larvae restores survival rates to wild-type levels, confirming the role of JNK in inducing cell death in the gut. *Candida* infection activates the fly innate immune system in a systemic response to which both host and pathogen factors contribute. Finally, the authors show that secreted aspartyl proteinases (SAPS) made by *Candida* are responsible for activating Toll immunity. Independently, a host-generated nitric oxide signal, which involves fly blood cells (haemocytes), also contributes to systemic immunity.

Implications and future directions

This host-pathogen model should enable the identification of factors that mediate the switch from GI infection to systemic candidiasia. In addition, the genetic tractability of *Drosophila* and its similarity to mammals with respect to innate immune defences and gut pathogenesis can be exploited to perform genome-wide RNAi experiments to uncover the host genes involved. Finally, this system can also be used to carry out high-throughput screens for pathogen factors that modulate host systemic immunity.

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