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Investigations of *Drosophila melanogaster* host defenses against *Aspergillus fumigatus* systemic infections

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présentée par :

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Biologie

**Investigations of *Drosophila*
melanogaster host defenses against
Aspergillus fumigatus systemic
Infections**

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摘要

背景: 果蝇是强大的遗传学研究模式生物,可以用来研究如发育、大脑功能、转录及感染等多个生理过程。

目的: 本研究的主要目的是通过实验更深入地了解宿主对抗人类机会致病菌烟曲霉 (*Aspergillus fumigatus, Af*) 的过程及机制。

材料与方法: 1、利用模式生物黑腹果蝇 (*Drosophila melanogaster*) 重构感染模型,并在此基础上开展研究。一系列 *Af* 产生的真菌毒素也被注射入果蝇加以研究。2、通过比较转基因 RNA 干扰 (RNA interference, RNAi) 果蝇感染 *Af* 后的生存率,开展大规模基因筛选以鉴定对感染敏感的品系。

结果: 1、仅有免疫反应相关的 Toll 通路基因突变体 *MyD88* 果蝇在低剂量 *Af* 孢子感染后生存率即出现明显表型,但在 *MyD88* 突变体果蝇体内, *Af* 并无扩散迹象。2、Restrictocin、verruculogen、fumitremorgin C、bromocriptine mesylate 注射入果蝇后仅对 *MyD88* 突变体果蝇具有毒性,而对野生型无效。Gliotoxin 注射后对两类果蝇具有无差别毒性, helvolic acid 和 fumagillin 注射后无毒性。3、在大规模遗传学筛选中,通过对 6,471 株果蝇表型的比较,发现了 241 株与宿主抗 *Af* 感染有关的基因。

结论: 1、这项研究揭示了在对抗 *Af* 及部分其分泌的毒素过程中,起主导作用的并非免疫反应而是对感染的恢复力。2、通过大规模筛选,我们发现了一系列对抗 *Af* 或其分泌的毒素的宿主恢复力基因。

关键词: 烟曲霉; 黑腹果蝇; Toll 通路; 感染恢复力; 真菌毒素

Abstract

Background *Drosophila melanogaster* is a genetic model organism that allows the exploration of many biological processes such as development, brain function, transcription or host defense against infections.

Objective The overarching goal of this work is to better understand host defenses against the human opportunistic fungus *Aspergillus fumigatus* (*Af*).

Method 1) An infection model has been reestablished in the genetic model organism *Drosophila melanogaster*. Some mycotoxins might be involved in pathogenesis which known to be secreted by *Af* also been tested by injection. 2) A large-scale genetic screen has been implemented to identify transgenic RNAi mutant lines susceptible to *Af* infection in survival experiments.

Result 1) Only flies mutant for the immune response Toll pathway gene *MyD88* succumb to the injection of a handful of conidia even though *Af* is unable to disseminate throughout its host. 2) Restrictocin, verruculogen, fumitremorgin C, bromocriptine mesylate differentially kill *MyD88* and not wild-type flies. Gliotoxin killed flies without difference, helvolic acid and fumagillin cannot kill flies. 3) 6,471 lines have been screened and 241 candidate genes identified, few of which are known to act in the immune response.

Conclusion 1) This work revealed that it is not the immune response that plays a cardinal role in host defense but its resilience capacity to the exposure to *Af* and some mycotoxins secreted by *Af*. 2) The large-scale screen has contributed to identifying

numerous genes involved in host resilience to *Af* and to some of its mycotoxins.

Key words: *Aspergillus fumigatus*, *Drosophila melanogaster*, *Toll pathway*, *Resilience to infection*, *Mycotoxin*

INTRODUCTION

***Aspergillus fumigatus*, a serious Public Health Threat**

Aspergillus fumigatus (*A. fumigatus*) is a rather ubiquitous saprotrophic fungus able to withstand high temperatures and to grow at 55°C. It thus is able to bypass the major barrier to fungal infections in warm-blooded animals: high body temperature. The dispersal form of *A. fumigatus* in the environment is the airborne conidium, the small size of which allows it to penetrate deeply in the airways of animals. Indeed, humans inhale daily hundreds to thousands of conidia (1), yet, mostly immunodeficient patients suffer from invasive aspergillosis. Of note, some cases of invasive aspergillosis affect apparently immunocompetent patients (2, 3). Invasive aspergillosis remains a major challenge to clinicians due to late diagnostic and high morbidity and mortality of this infectious disease.

A. fumigatus has a complex genome that allows it to adapt to varied environmental conditions and also to host defenses as well as to antifungal drugs (4). For instance, a range of efflux pumps and transporters protects it against toxic compounds, including possibly azoles used to cure invasive aspergillosis (5). The virulence strategies of *A. fumigatus* are being deciphered and include intracellular germination of the ingested conidium that leads to mechanical lysis of the phagocyte. A characteristic of *Aspergillus* species is their rich secondary metabolism that allow them to produce a rich variety of metabolites, including mycotoxins. It has been hypothesized that the *in vivo* production of specific secondary metabolites by *A. fumigatus* contributes to its

pathogenicity, particularly during hyphal growth. Gliotoxin (6), fumagillin (7), helvolic acid (8), restrictocin (9), verruculogen (10), ergot alkaloids (11) and fumitremorgins (12) are mycotoxins secreted by *A. fumigatus* (Fig 1).

Gliotoxin. The biological activity of gliotoxin is based on an internal disulfide bridge that can bind and inactivate proteins *via* a sulfide/thiol exchange as well as ROS produced by redox cycling between oxidized and reduced forms of the toxin. *In vitro* studies of gliotoxin function have identified multiple immunosuppressive activities including: i) an inhibition of macrophage phagocytosis, mitogen-activated T-cell proliferation, mast cell activation, and cytotoxic T-cell responses (13-17); ii) the suppression of immune cell reconstitution following sublethal irradiation (18); iii) the slowing of ciliary beat frequency and induction of epithelial cell damage (19); iv) the induction of apoptosis in lymphocytes, phagocytes, dendritic cells, liver cells, fibroblasts, and cancer cells (16, 20-26). Previously reported mechanisms of apoptosis induction include the induction of TNF-mediated cell death, activation of caspase-3 and ROS, inhibition of NF- κ B activation, and activation of Bak, which in turn activates ROS production, mitochondrial pore formation, and cell death (27-29). Gliotoxin has also been shown to inhibit antigen presentation by monocytes and dendritic cells to effector T cells, limiting the subsequent expansion of an antigen-specific adaptive response. Furthermore, gliotoxin may prevent the formation of the NADPH oxidase complex in neutrophils (30, 31). Together, these studies reveal the broad nature of gliotoxin immunosuppression by preventing cellular effector functions or inducing cellular apoptosis. Up to now, gliotoxin is considered as the

most important mycotoxin produced by *A. fumigatus*.

Fumagillin, which targets methionine aminopeptidase-2 (32, 33) has been used to treat corneal microsporidial keratitis (34) and has also been identified as an angiogenesis inhibitor (35, 36). It can also be used to treat microsporidia-infested *Drosophila* stocks.

Helvolic acid is a nortriterpenoid antibacterial compound that inhibits translation elongation factor 2 and the oxidized low-density lipoprotein metabolism of bacteria (37, 38).

Restrictocin is a protein produced by Aspergilli that belongs to the superfamily of ribonucleases and specifically cleave 28S ribosomal RNA at a specific loop (39, 40). These fungal ribotoxins are being developed into anticancer drugs (41).

Verruculogen and fumitremorgins are prenylated indole alkaloid metabolites of Aspergilli which are tremorogenic. Their chemical structures are similar: fumitremorgin B can be converted into verruculogen by the FtmOx1 enzyme (42). They function similarly and act by reducing GABA levels in the central nervous system thereby inducing tremors, as GABA is the major inhibitory mediator of the nervous influx (43, 44); they also inhibit the M phase of cell cycle to inhibit the cell proliferation (45).

Ergot alkaloids are produced by many fungal species (46). One ergot alkaloid, festuclavine, interferes with several mammalian regulatory systems via its ability to bind to serotonin, to dopamine, and to α -adrenaline receptors (47). Although genes regulating the production of clavine ergot alkaloids have been identified for *A.*

fumigatus, the role of these metabolites in the pathogenesis of IA has yet to be explored (48-50).

Detection of *A. fumigatus*

Pattern recognition receptors (PRR) can detect some invariant features shared by groups of microorganisms. Common components of fungal surfaces are β -glucans, and mannans. There are several PRRs involved in detection of *A. fumigatus*: secreted PRRs, which comprehends pentraxin 3, collectins, and complement factors; endocytic and transmembrane PRRs, which encompasses DC-SIGN, the mannose receptor, and Dectin-1; signaling PRRs, TLRs, Dectin-1, NOD-2. Then, these PRRs activate through the CARD9 adapter transcription factors belonging to the NF- κ B family to induce the expression of cytokines and effector genes.

Innate immune effectors against *A. fumigatus*

Here, I will focus on mammalian factors that have been much more studied (Fig 2). In immunocompetent hosts, most of the invading *A. fumigatus* were recognized and cleared by innate immunity (51, 52). The major entry route of conidia of *A. fumigatus* to get into the mammalian body is via the airways. Most of the inhaled conidia are eliminated by sneezing, cough, and ciliary beating of the mucous epithelium. As countermeasures, *A. fumigatus* secretes a series of proteinases which will damage the smooth surface of tissue to make the attached conidia more difficult to be cleared. Some of the secondary metabolites of *A. fumigatus* will impair the ciliary beating (11,

19, 53, 54). In addition, epithelial and endothelial cells have been shown to internalize conidia, serving as putative foci of infection (55, 56). In animal models, penetration at the epithelial level is common (57, 58). The research on the function of the lung epithelium against *A. fumigatus* as the first barrier of innate immunity is well-developed. The alveolar macrophages will efficiently engulf and kill the conidia that have reached the alveoli. Macrophages are able to take up dormant or swollen spores, but kill only swollen spores. The dormant spores become active and swell in the phagolysosomal compartment upon ingestion, a major mechanism for macrophages to eliminate all invading microorganisms. Macrophages cannot kill the germinated spores that have formed hyphae (59). Neutrophils can release the contents of their granules into the extracellular medium to eliminate the extracellular *A. fumigatus* hyphae. The recruitment of neutrophils is a cardinal process to eliminate *A. fumigatus*. Lung surfactant plays a protective role against pathogens before the spores of *A. fumigatus* arrived to the alveoli. Some studies reported that the hydrophilic surfactant proteins A and D enhanced agglutination, phagocytosis, and killing of conidia of *A. fumigatus* by alveolar macrophages and neutrophils (60).

Antimicrobial molecules produced by the cells of the upper and lower airways are also involved in the inhibition and elimination of *A. fumigatus*: hypochloric acid (HOCl), superoxide anion radical (O_2^-), Secretory Leukoprotease Inhibitor (SLPI), pre-elafin trappin-2d serine protease inhibitors, lactoferrin, ubiquicidin, elastase, cathepsinG, chitinases, Drosomycin-like defensin (DLD). These molecules may act directly as endogenous antibiotics, or indirectly, by facilitating the elimination of

infectious agents by phagocytes. Phagocytosis is central to eliminate invading *A. fumigatus*. Oxidative mechanisms involving reactive oxygen species and reactive nitrogen species derived from nitric oxide (NO) are important effectors within the phagolysosomal vacuole. The enzymes involved in the production of reactive oxygen species during phagocytosis include nicotinamide adenine dinucleotide phosphate (NADPH) and oxidase myeloperoxidase (MPO), responsible for the production of superoxide anions and hypochloric acid (HOCl), respectively. Of note, the former diffuses farther away than the latter and therefore induces more collateral damage to the host cell. An *in vitro* study has shown that the macrophages from mice lacking NADPH oxidase (p47phox^{-/-}) lose their fungicidal activities, which implied that reactive oxygen species are essential for macrophages to kill fungi (59). In another *in vivo* investigation, the absence of MPO led to higher mortality rates in *A. fumigatus* infected mice (61). These findings confirm those of an *in vivo* study demonstrating that mice lacking NADPH oxidase are more susceptible to infection with *A. fumigatus* (61). They explain why invasive pulmonary aspergillosis is the leading cause of death in patients with chronic granulomatous disease (CGD), a disease characterized by the absence of NADPH oxidase from neutrophils and macrophages. However, the production and efficacy of reactive nitrogen species during *A. fumigatus* infection remain to be demonstrated. Studies of macrophages from mice lacking NO synthase (iNOS ^{-/-}) have shown that the fungicidal activity of macrophages is independent of the production of NO derivatives (59). The SLPI and pre-elafin trappin-2d serine protease inhibitors can be produced by epithelial cells, macrophages and neutrophils,

which anticipate the process of maintenance of the protease/antiprotease balance in the respiratory tract. A study identified their activity against *A. fumigatus* in particular (62). Lactoferrin produced by neutrophils and epithelial cells can inhibit the proliferation of *A. fumigatus* by binding to an element essential for its growth, iron (63). *In vitro* research that compared the activity of several antimicrobial peptides confirmed the antifungal effects of lactoferrin (64). This work suggested the possible use of peptides derived from lactoferrin or from ubiquicidin, another antimicrobial peptide produced by respiratory epithelial cells, as novel agents for the treatment of aspergillosis. Elastase and cathepsin G are stored in azurophilic granules of neutrophils. These serine proteases have been reported to be active against *Aspergillus* and mice lacking either one of these enzymes are more susceptible to *A. fumigatus* infection (65). Chitinases, produced by epithelial cells and macrophages, are endo- β -1,4-N-acetylglucosaminidases capable of degrading chitin, an essential component of the cell wall of *A. fumigatus* (66). Although it was shown that DLD, the human homolog of the *Drosophila* antimicrobial peptide Drosomycin has a specific antifungal activity on filamentous fungi such as *A. fumigatus*, it appears to be mostly present in the skin (67).

Thrombosis and hemorrhagic infarction occurred when hyphae invade the vascular system. In humans, platelets attach to the cell walls of the invasive hyphal form of *A. fumigatus* and become activated during attachment to hyphae (68). Several anti-*Aspergillus* functions, including direct cell wall damage and enhancement of neutrophil-mediated fungicidal effects, have been associated with platelets.

Adaptive immune response against *A. fumigatus*

Some pattern-recognition receptors (PRR) at the surface of dendritic cells will favor the orientation of the immune response towards Th17 and Th1 cellular immune responses, involving the two phagocytes described in the previous part, respectively neutrophils and macrophages. Even if the cellular immune response is the most adapted to fight against fungal infections, the humoral response, with production of antibodies, may be a complementary facet of the adaptive immune response, as it was also shown with the demonstration of the antibody-mediated inhibition of the hyphal development and metabolic activity of *Aspergillus* (69).

Host defense: resistance and resilience to infections

Host defense against infection has evolved to preserve living organisms from infections and parasitism. A much-studied aspect of host defense is immunity, which encompasses both innate and adaptive arms. It involves directly fighting off potential pathogens and parasites by mobilizing an armamentarium of distinct weapons, from antimicrobial peptides to phagocytosis by specialized effector cells such as macrophages and neutrophils and the selection of high affinity antibodies and receptors carried by immune cells that allow the specific detection and elimination of pathogens or infected cells. Nevertheless, evolutionary pressure aims at perpetuation of the species to the next generation, and still, living organisms are often parasitized, thus underlining the limits of immune defenses, even when they encompass adaptive

immune responses. Furthermore, our current understanding of immunity does not satisfactorily account for healthy carriers of pathogens (70). In our own studies of intestinal infections of the model genetic organism *Drosophila melanogaster* by the Gram-negative entomopathogenic bacterium *Serratia marcescens* (*Sm*), we encountered an unexpected phenomenon: most of the candidate genes we identified in a genome-wide survival screen to *Sm* ingestion were not obviously involved in innate immunity and we could demonstrate that at least some of these were required to maintain the homeostasis of the midgut epithelium (71), a phenomenon that we called **resilience** to mirror the active repair mechanisms that are at work during infections (72). Actually, a rather similar observation had already been made by plant pathologists at the eve of the 20th century (73). They found that some wheat cultivars were still able to produce relatively abundant crops despite heavy rust infections. This property was named tolerance and is now also found in the animal literature (70, 74, 75), even though it is a source of confusion with immunological tolerance and tolerance to the microbiota. Furthermore, it etymologically implies a rather passive ability to endure infection and does not account for the active processes that are involved (72). The field of tolerance/resilience is just starting to be deciphered in molecular terms. The first studies in animals considered tolerance more in terms of ecological immunity, in keeping with plant immunology. For instance, infecting several strains of mice with distinct strains of *Plasmodium chabaudi* revealed genetic variations in resistance and tolerance (76). The infection of red blood cells by the *Plasmodium* parasites leads to the release of free heme in the blood, the toxicity of

which is alleviated by heme oxygenase 1, which protects hepatocytes from TNF-induced apoptosis (77). More recent studies have further deepened our understanding of this mechanism (78) also illustrated the importance of tissue-repair in the case of double viral and bacterial infections (79). We want here to briefly relate an outstanding piece of work that epitomizes the concept of resilience (80). Septic shock is a major clinical problem that kills hundreds of thousands of patients each year in hospitals. It is caused by a systemic inflammatory reaction triggered by a "cytokine storm" resulting from the detection of an infection and that provokes multiple tissue and organ failures. Antibiotics treatment is often inefficient, as it is given too late. The authors have shown that treating mice with anthracyclines in a model of septic shock protects them. Anthracyclines trigger the DNA damage response and ultimately autophagy. The induction of autophagy in lungs is sufficient to protect mice from sepsis, even when septic shock has already developed. Remarkably, in this cecal ligation and puncture model of sepsis, anthracycline treatment functions without decreasing the bacterial burden. Thus, this study illustrates how triggering a stress response pathway contributes significantly to host defense through its resilience arm. *Thus, whereas resistance aims to directly attack pathogens, we define resilience as being the mechanisms that allow a host to withstand and/or actively repair damages inflicted either by the pathogen or by the host's own immune response.*

In practical terms, a host strain will be deemed to be more "resilient" than another if it presents a better fitness when confronted to a similar pathogen load, usually measured

in colony forming units (81). However, care should be taken to establish that the microbe is in the same physiological condition in both strains, *i.e.*, that they express the same virulence programs, a condition that has not been checked in most studies (82). Indeed, one might think that the host may either downregulate virulence programs or alternatively increase the virulence of the invading pathogen, for instance by producing antimicrobial peptides (AMPs) that are sensed by the bacteria (83, 84). Resilience is unlikely to affect the evolution of microbes in the way resistance to the immune response has been selected for in pathogens because it is not thought to exert pressure on the pathogen (81). In summary, resilience may be thought of as the intersection between host defense against infectious disease and homeostasis mechanisms of the host, at levels ranging from the cell to the whole organism. It may take unexpected forms as exemplified by our recent characterization of the enterocyte purge, which is triggered by the exposure of the intestinal epithelium to bacterial pore-forming toxins, a mechanism that ultimately involves the organism as a whole (85).

The *Drosophila melanogaster* genetic model organism

The genetic study of infectious diseases in mammalian models is severely hampered by cost, space, and ethical considerations. Genetic model organisms provide interesting alternatives as they allow direct large-scale screens. In this way, "public" virulence factors can be identified, that is factors required for pathogenesis in multiple hosts. They also allow the identification of "private" virulence factors (that is

virulence factors required only to infect successfully the model organism and not other hosts), which have then to be understood in the context of the peculiarities of the host immune system. More generally, genome-wide approaches are a workable option in which it is possible to ask such broad questions as which genes are required for host defense against a given fungal pathogen, in essence a functional genomics approach that can be implemented only in model genetic organisms. Most of our knowledge on the biology of model organisms such as *D. melanogaster* or *Caenorhabditis elegans* derives directly or indirectly from unbiased genetic screens. For instance, our current understanding of *Drosophila* innate immunity is largely dependent on the completion of genome-wide genetic screens aimed at understanding the humoral immune response (86). The Strasbourg laboratory has largely participated to this endeavor (87-91).

The fruit fly *Drosophila melanogaster* is one of the most studied metazoan, thanks to over a century of research (92). It is small, grows rapidly, produces an important offspring, and is relatively cheap to raise. Its genome, as well as that of more than 20 other *Drosophila* species, is available and provides a wealth of evolutionary information. With more than a century of investigations, *Drosophila* is a potent genetic model that allows easily characterizing biological processes from the scale of the molecule to that of the whole organism, including population biology as well as evolutionary studies. It has led to many major breakthroughs in our understanding of life, for instance to cite only Nobel Prizes, the chromosomal theory of heredity (Thomas Morgan, 1933), the discovery of the mutagenic effects of X-rays (Hermann

Muller, 1946), the identification of developmental genes (Ed Lewis, Christiane Nüsslein-Volhard, Eric Wieschaus, 1995), the activation of innate immunity (Jules Hoffmann, 2011), and the molecular mechanisms of biological rhythms (Jeffrey Hall, Michael Rosbach, Michael Young, 2017). Besides the large body of knowledge generated by the community and easily accessible on the Flybase web site, a large palette of genetic tools is available to express, inactivate almost any gene in a time-dependent, cell type and tissue-specific manner, for instance using the Gal4/UAS system combined with dsRNA or shRNA transgenes (93). Gene products can be further tagged or edited using a variety of techniques, including CRISPR-Cas9 (94-96). Of note, our Institute in Guangzhou is developing a CRISPR-Cas9 mutant platform. Reporter transgenic lines are available, as well as Gal4 drivers that are expressed in specific tissues, including distinct neuronal sets (97, 98). Importantly, many stocks can directly be ordered from stock centers or sent by investigators upon request, a tradition that has made the fly field so strong. The RNAi methodology is especially well-developed and allows bypassing developmental lethality through the use of conditional expression. The first strategy was to generate transgenic long double RNA hairpins that would target the endogenous transcripts through the dsRNA degradation pathway. Two genome-wide collections are available at the Vienna *Drosophila* RNAi Center (VDRC) (99). A more recent strategy has been developed by Dr. Ni and Perrimon and involves shRNAs that trigger the miRNA pathway (100). A large collection is housed partially at Tsinghua University and the whole collection is available at the Bloomington stock center in the US.

Innate immunity in *Drosophila*

Epidermal defense is the first line of host defense in *Drosophila*. The exoskeleton of insect is constituted of cuticle that is secreted by the underlying hypoderm, is an efficient physical barrier to prevent environmental microbes from invading the aseptically body-cavity of insect. Except for entomopathogenic fungi and some nematodes carrying invasive bacteria as biological weapons, most environmental microbes cannot pierce the protection of the cuticle (101, 102). The cuticle also covers the respiratory system and large parts of the intestinal tract. In addition, the entrances of the respiratory tract are protected by spiracles which prevent the microorganisms from entering into even the major tracheal trunks (72). Thanks to this elaborate protection system, up to now, there are no reported infection models of the *Drosophila* respiratory tract. Local AMP expression, ROS production and TEPs expression are also play important roles in epidermal defense (103, 104) .

In wild environment, damage to the exoskeleton of insects is commonly observed. When microorganisms are introduced or invade the cavity of *Drosophila*, they will face three major arms of the insect host defense: the melanization response, the cellular response, and the systemic humoral immune response (Fig 3).

Coagulation and melanization are immediate immune responses in *Drosophila* which happened after the physical puncture of the cuticle (Fig 5). Coagulation consists in the formation of clots that limit the loss of hemolymph, and the next step the melanization and epithelial movements to heal the wound (105) . Melanization is the process of

synthesis of melanin, which plays an important role in wound healing, encapsulation, control of the dissemination of microorganisms, and the production of toxic reactive oxygen species intermediates that are speculated to kill invading microorganisms (106, 107). The key enzyme of melanization is phenol oxidase (PO) which is activated by the cleavage of its pro-form pro-phenoloxidase (PPO). There are three PPOs (PPO1, 2, and 3) in *Drosophila*, PPO1 and 2 are produced by crystal cells that contribute to hemolymph melanization in larvae, whereas PPO3 is produced by lamellocytes involved in the encapsulation of parasitic wasp eggs during larval development (108, 109). In adults, unpublished data from Lucas Walzer suggest that some 8% of adult hemocytes express PPOs. The initiation of a protease cascade leads to the cleavage of enzymatically inactive PPOs into active PO and is performed by at least three serine proteases: MP1, MP2/Sp7/PAE1, and Hayan (110-113). The molecular mechanism involved in triggering PO activation remains poorly understood in *Drosophila*, and there are at least two ways to initiate the cascade: one is wounding that possibly activates Hayan to cleave PPOs, the other is Toll pathway-dependent way to activate Hayan to cleave PPOs, there also has a possibility is that an attack complex forms targeted by PRRs and bringing PO in proximity to the pathogen (113-115) (Fig 2). Former research showed PPOs play different roles in against infection, on the one hand there is the formation of a melanic plug at the point of injection that involves both PPO1 and PPO2 and on the other there is a pathogen killing activity, which in the case of *Staphylococcus aureus* depends on PPO1. Of note, for *Pseudomonas aeruginosa* and *Metarhizium anisopliae* (*M. anisopliae*), likely *A. fumigatus*, it is

PPO2 and PPO1 are required to neutralize the proliferation and the virulence of pathogens, whereas PPO1 is only required to control the virulence (pathogen proliferate but no longer kill the HOST) (116, 117). During the synthesis of melanin, reactive oxygen species (ROS) and other cytotoxic metabolic intermediates *eg.* quinones are produced and may participate in killing the pathogens (118).

The cellular response mainly involves phagocytosis and encapsulation, both of which are mediated by hemocytes. *Drosophila* hemocytes can be divided into three types which are plasmatocytes, crystal cells, and lamellocytes, the latter type being normally not found in normal development but differentiates upon the injection of a parasitic wasp egg in larvae (119). The wasp egg will be encapsulated first by plasmatocytes and then by lamellocytes prior to the the melanization of the resulting capsule. The parasite egg will also be killed by ROS and other cytotoxins produced in melanization cascade (120). Phagocytosis is mainly performed by plasmatocytes, which will respond to and engulf both microorganisms and apoptotic cells. There are several receptor proteins playing roles in phagocytosis, which include the scavenger receptor family (dSR-CI), the EGF-domain protein Eater, and other receptors of the Nimrod family. Encapsulation is another cellular response which mediated by lamellocytes in *Drosophila* larvae.

The Toll pathway was initially found as an early *Drosophila* embryonic development pathway (121). A genetic analysis of dorsal-ventral patterning of the embryo has defined the series of genes that constitute the Toll-Dorsal pathway (122). Because Dorsal is a *Drosophila* homolog of NF- κ B and that NF- κ B is a central player of

mammalian innate immunity and inflammation, the *Drosophila* Toll-Dorsal pathway has been hypothesized to also play a role in *Drosophila* immunity parallel to its developmental function. Although in 1995, Toll (Toll-1) had been identified as an immune activator *in vitro* (123), the demonstration of the Spätzle-cactus cassette *in vivo* in the antifungal response from *spätzle* to *cactus* was published one year later (124). One Toll-like receptor (TLR) was shown to function in mammalian innate immunity one year later (125, 126). In *Drosophila*, the activation of the Toll pathway is different from that occurring in mammals, in which TLRs directly sense some conserved structures of the microbial cell wall from microorganisms such as the lipopolysachharide of the Gram-negative bacterial cell wall. The *Drosophila* microbial sensors are divided into two family by their structures: the peptidoglycan-recognition proteins (PGRPs) and the Gram-negative binding proteins (GNBPs). In Toll pathway, GGBP1 sensing Gram positive bacteria by Lysine-type peptidoglycans (Lys-type PGNs) and PGRP-SA cooperate to detect some Lys-type PGNs (87, 127). GGBP3 plays the key role in sensing the β -(1,3)-glucans from fungal cells (115). The GGBP3 and PGRP-SA/GGBP1 sensors activate a proteolytic cascade that includes the MoDSP, Grass, and Hyan and Persephone, the latter two appearing to function redundantly (113, 128-130). There is another sensing system independent from the microbial cell wall component sensors by Persephone, which senses the proteolytic activity of secreted microbial virulence factors (88, 129, 131). The signal from these receptors will active the Spätzle-Processing Enzyme (SPE) to cleave the proSpätzle into Spätzle. Spätzle is the ligand of Toll receptor (132, 133).

In *Drosophila*, the Toll pathway plays the most important role in the humoral response against fungi and some Gram-positive bacteria. Toll is a transmembrane receptor (134). The intracytoplasmic region of Toll is Toll-IL-1R (TIR) domain, which complexes with three main intracytoplasmic partners: MyD88, tube and pelle (135, 136). These partners all have death domain region, MyD88 and tube are adaptor proteins, pelle is a serine-threonine kinase. The Toll receptor-adaptor complex signals to a latent transcriptional factor of the NF- κ B-Rel family of inducible transactivators. The ankyrin-repeat inhibitor protein Cactus associates with this factor, which dissociates from the factor upon its Toll signal-dependent phosphorylation (137). The transcriptional factor is made by two Rel proteins: Dorsal and Dorsal-related immunity factor (Dif), the former playing a role in development and not so much in the immune response (138). After Cactus's dissociation and proteolysis, Dif gets into nucleus to activate the transcriptions of several genes during the immune response. The expression of some potent antimicrobial peptides (AMPs) is regulated by the Toll pathway: Drosomycin and Metchnikowin, and possibly Cecropins have antifungal activity (139-141).

The Immune deficiency (IMD) pathway is another important component of the humoral response against Gram-negative bacteria. The activation of IMD pathway is initiated by the recognition of the di-aminopimelic acid (DAP)-type of PGN present in the cell wall of Gram-negative bacteria as well as Gram-positive bacilli. Upon binding DAP-PGN, PGRP-LC recruits IMD and thereby initiates an intracellular signaling pathways that ultimately activates the NF- κ B Relish factor in two complementary

ways: 1- cleavage through the DREDD caspase thus liberating it from the ankyrin-repeat cytoplasmic anchor present in its C-terminal domain and thereby allowing nuclear uptake; 2- phosphorylation of the N-terminal domain by the I- κ B Kinase complex that enables the transcription initiation transactivation properties of this transcription factor (142). The IMD pathway regulates the expression of several AMPs, for example Dipterucin and Drosocin (Fig 5). It also activates the expression of other AMP families such as Cecropins, Attacins, and Defensin, with an input from the Toll pathway (143, 144). Indeed, upon persistent infections with pathogens, the Toll pathway gets activated even by Gram-negative bacteria, either through the secretion of proteases sensed by Persephone or through PGRP-SA, which does bind DAP-PGN, albeit with a lower affinity than LYS-PGN.

Fungal pathogens in the *Drosophila* model

As mentioned above, the Toll pathway provides a major defense against fungal infections in flies. One major AMP controlled by the Toll pathway is Drosomycin, which is synthesized in very high concentrations by the fat body (100 μ M). It is active against filamentous fungi such as *Aspergillus fumigatus*, but inactive against *Candida* and entomopathogenic fungi (88, 140, 145). Several studies have used Toll pathway mutants to investigate the virulence of several fungi (124, 146, 147). For instance, the *Candida glabrata* (*C. glabrata*) pathogenic yeast is able to kill Toll pathway mutants but not flies deprived of a cellular immune response (148). Interestingly, hemocytes form the major remaining host defense in Toll pathway mutants. Of note, *C. glabrata*

injected in wild-type flies are not cleared and remain present, albeit with no net proliferation, suggesting that the immune response against such yeasts is fungistatic (148). The immunocompromised Toll mutant as background was used to identify relevant virulence factors among 500 *C. glabrata* mutants generated within a European Research network and correlated with findings in mice (149). A limitation of this approach is that it relies on the use of human and not insect-specific pathogens so that the fungi are introduced in the hemocoel by septic injury. In contrast, spores of entomopathogenic fungi such as *Beauveria bassiana* (*B. bassiana*) and *M. anisopliae* (now called *M. robertsi*) penetrate insects by enzymatically boring a hole through the cuticle in a natural infection model in which conidia are deposited on the surface of the insect cuticle (150). These fungi have been widely studied for their use as pest control agents in agriculture and are also used to kill mosquito vectors of malaria (151, 152). The extensive literature on these fungi has relied mostly on insect pests as infection models. In contrast, the *Drosophila* model has been used mostly to analyze the host response. Two major host defenses are required to slow down *M. anisopliae* infection, the Toll pathway and the cellular immune response, in accordance with results obtained with *B. bassiana* (88, 124, 153). Historically, the first stage of the analysis of the pathogenicity of these entomopathogenic fungi was biochemistry on large insects in the late 80's and early 90's. It led to the characterization of proteins present on spores and blastospores, of the secreted chitinase and protease enzymes required to penetrate the cuticle, and of toxins such as *B. bassiana* Beauvericin or *M. anisopliae* destruxins. More recently, EST libraries have been established using

spores incubated in hemolymph and the genome of fungi such as *B. bassiana* and *M. anisopliae* is now available (101, 102). This should pave the way for a thorough genetic investigation of these fungi, which will be useful for our long-term goal of studying host-pathogen relationships of *M. anisopliae* in the *Drosophila* model.

Like in mammals, $\beta(1,3)$ glucans appear to be the major cell wall constituent detected by the fly immune system, although in insects this is achieved by a circulating Pattern Recognition Receptor, GGBP3, that triggers humoral responses (AMPs, melanization) whereas in mammals the Dectin lectin is membrane-bound and activates phagocytosis as well as signaling (88, 154). The cell wall composition varies according to the developmental stage: both *C. albicans* and *A. fumigatus* yeast forms, respectively conidia, display distinct types of glycans as compared to hyphae (155). Furthermore, the size of the fungus is an important parameter for phagocytosis: hyphae render the fungus too large to be internalized by phagocytes (156). Interestingly, AMPs active against filamentous fungi (*e.g.*, Drosomycin) but not against yeasts have been identified, although a cluster of some 10 potential AMPs has been identified at locus 55C and is likely to mediate much of the effect of the Toll pathway (157). The theme of concealment of molecules eliciting the immune response is also at play in *M. anisopliae*, the blastospore of which is coated by Mcp-1 (158), a collagen-like protein, which in *Drosophila* is induced only in the injection model and makes the fungus less susceptible to the cellular immune response. Studies on the antifungal response in mammals have focused on the cellular immune response, mediated by two key phagocyte populations, short-lived neutrophils able to elaborate a ROS-burst and

macrophages. Whether *Drosophila* adult hemocytes are akin to macrophages or neutrophils can be debated and it appears that a ROS-burst does not take place within the phagosome (159, 160). Whether in mammals or in insects, resilience remains an understudied area of research. A couple of genetic approaches (direct screen of transposon insertion lines, probing of polymorphic lines isogenized from natural populations) implemented using *M. anisopliae* has identified a spate of loci involved in resistance/susceptibility to this fungus (161, 162). Only one line appeared to involve resilience/tolerance, as flies from that line succumbed in the absence of significant fungal growth during the final stage of the disease. However, a major limitation of these studies was that it required the genes under study to be viable, which excludes most strong alleles of signaling pathways.

Aspergillus fumigatus* infection on *Drosophila

In contrast to these natural pathogens of *Drosophila*, *Aspergillus fumigatus* is a fungus of medical interest and although it is likely that flies encounter it, it does not appear to be a major pathogen, as wild-type flies are resistant to this infectious challenge. *A. fumigatus* is the fungus that was initially used to demonstrate that Toll pathway mutants are sensitive to fungal infections, with a now famous picture on the cover of Cell showing a diseased fly with hyphae coming out of the thorax of the cadaver. Of note, high concentrations of *A. fumigatus* conidia were used for inoculation. Because Drosomycin is induced to very high concentrations (0.1 μ M) within 24 hours under the control of the Toll pathway, it was thought that Drosomycin is the Toll pathway effector that controls *A. fumigatus* in wild-type flies (140).

Indeed, the overexpression of *Drosomycin* from a transgene provides some protection against *A. fumigatus* to a Toll pathway mutant that is otherwise highly susceptible to this infection (145). Finally, a mutant line devoid of several AMP genes, including *Drosomycin*, exhibits some susceptibility to an *A. fumigatus* challenge (163).

Unexpectedly, few studies have been performed to investigate other host defenses and some have been conducted on other insects such as *Galleria mellonella* (164-166). A study reported that Toll pathway mutants could be infected by three different routes, injection, natural infection, and feeding (167), a somewhat unexpected finding since hardly any studies have documented a fungal infection through the *Drosophila* digestive tract, which is strongly protected by cuticle or its peritrophic matrix. Furthermore, it is not readily apparent that *Aspergillus* has the ability upon germination on the cuticle to form an appressorium, the structure used by fungi to invade the epidermis of their hosts. Toll pathway mutants have been used in a limited manner to identify some virulence factors such as gliotoxin, siderophores or volatile compounds emitted by the pathogen (168). Six mutant strains displayed a similar degree of attenuated virulence in mice and in flies (169). In the case of the gliotoxin deletion strain, it is noteworthy that this strain was less virulent in mice immunosuppressed by corticoids but not in neutropenic mice (170). Like in flies, this result is somewhat paradoxical in that one target of gliotoxin is the activation of the NF-kappaB pathway, which is suppressed both in Toll flies and mice exposed to glucocorticoids; gliotoxin should therefore be dispensable as a virulence factor in these models.

GOALS of this thesis

A medical doctor by training, I have a strong interest in infectious respiratory disease. As *Drosophila* is a powerful model, I decided to join SFHI at Guangzhou Medical University to get the opportunity to study *A. fumigatus* infection in fruit flies, as I am convinced that we are far from a thorough understanding of its pathogenesis. Indeed, *A. fumigatus* is able to infect apparently immuno-competent patients. Conversely, some patients endure an unusually high fungal burden yet exhibit rather mild symptoms and survive the infection (personal communication with Axel Brakhage) . I thus redeveloped a coherent infection model in *Drosophila*, a process that led me to revisit the current understanding of its host defense against this fungus. An overarching goal of the host team is to perform a large-scale genetic screen to identify host factors involved in the *Drosophila* host defense against fungal infections.

Hence, there will be three parts in this thesis: the first part is about a project which revealed that *MyD88* plays an essential role in the resilience to *Aspergillus fumigatus* infection in *Drosophila melanogaster* but does not appear to be required to prevent the dissemination of the fungus in the infection paradigm I developed; the second parts reports our efforts in implementing a large-scale genetic screen, *A. fumigatus* being one of the five pathogens tested for the whole large-scale screen performed in parallel at the Sino French Hoffmann Institute; finally I will report a preliminary study on the CycK/Cdk12-Nrf2 axis, which was one of the first interesting candidate identified in the large-scale screen.

Fig 2

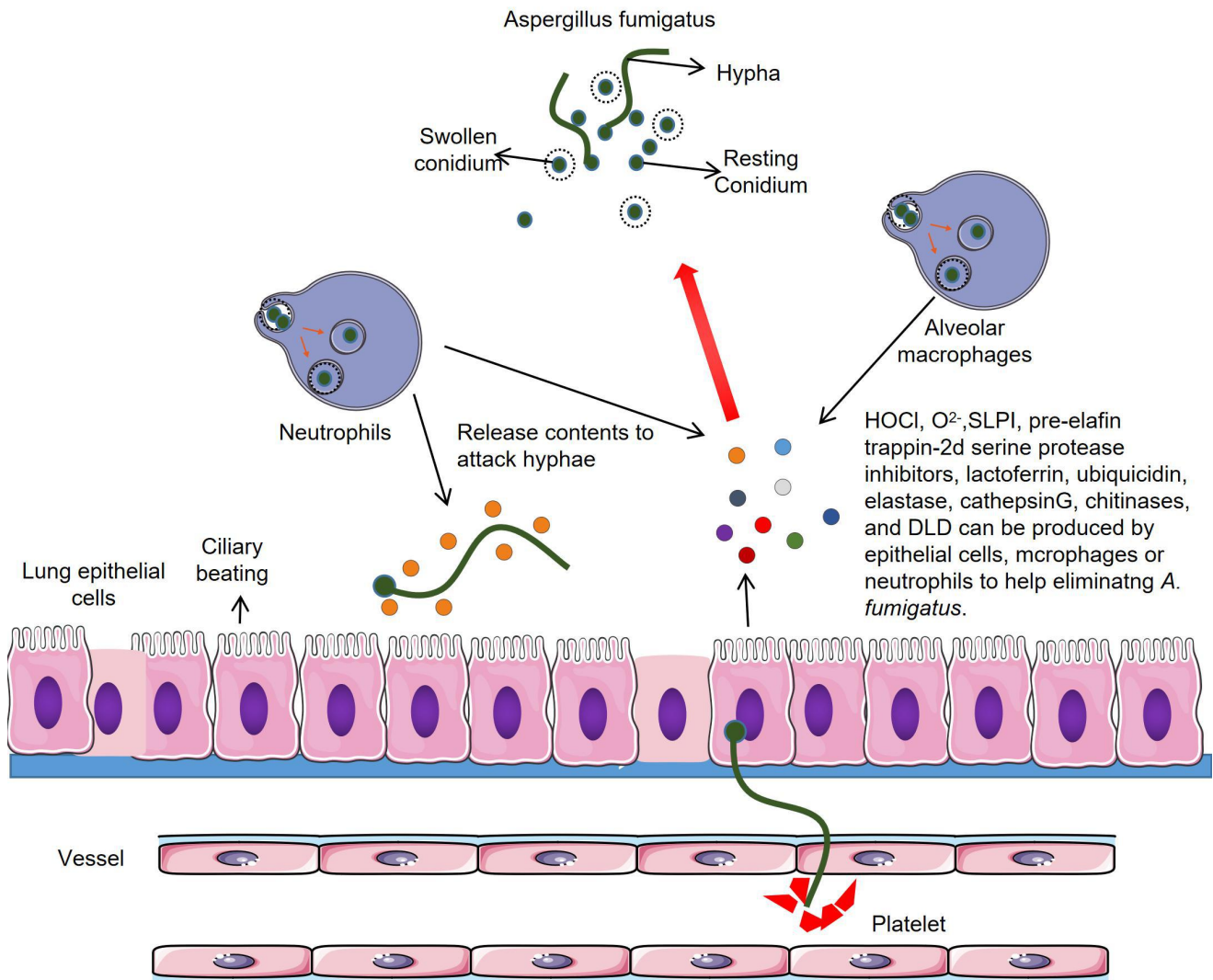


Figure 2: Mechanisms of innate immunity against *A. fumigatus* in mammals. There germination of *A. fumigatus* starts from resting conidia that then swells prior to the growth of hyphae. Most of the inhaled conidia are eliminated by ciliary beating of epithelial cells in the airway or coughing, the conidia in alveoli are engulfed by alveolar macrophages and neutrophils. Neither alveolar macrophages nor neutrophils can phagocytose hyphae, however neutrophils can release its contents to attack hyphae. Alveolar epithelial cell, alveolar macrophages and neutrophils produce a series of products to help detect and kill the conidia and hyphae of *A. fumigatus*. Platelet attach to the hyphae that invade vessel and damage the cell wall of *A. fumigatus* directly also enhance the neutrophil-mediated fungicidal effects. *HOCl*, hypochloric acid; O_2^- , superoxide anion radical; *SLPI*, secretory leukoprotease inhibitor, *DLD*, Drosomycin-like defensin.

Fig 3

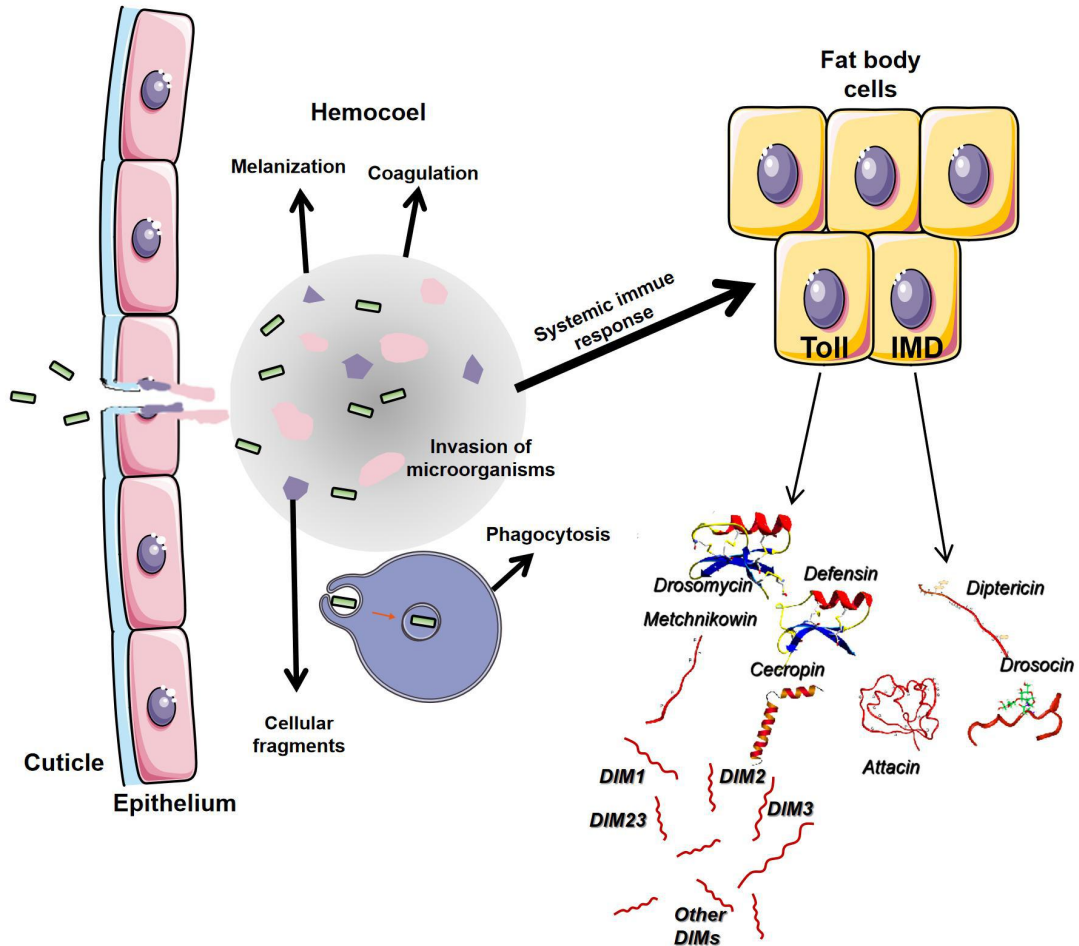


Figure 3: Overview of the *Drosophila* innate immune response to systemic infections. Coagulation and melanization are immediate immune responses in *Drosophila* that occur after the physical puncture of the cuticle. Cellular responses in adult *Drosophila* essentially is phagocytosis performed by a category of hemocytes, the plasmatocytes. The systemic immune response is triggered by the sensing of pathogens by sensors that activate NF- κ B mediated immune pathways, eg. Toll or/and IMD pathway, to produce the antimicrobial peptides. *DIM*, *Drosophila* immune induced molecular.

Fig 4

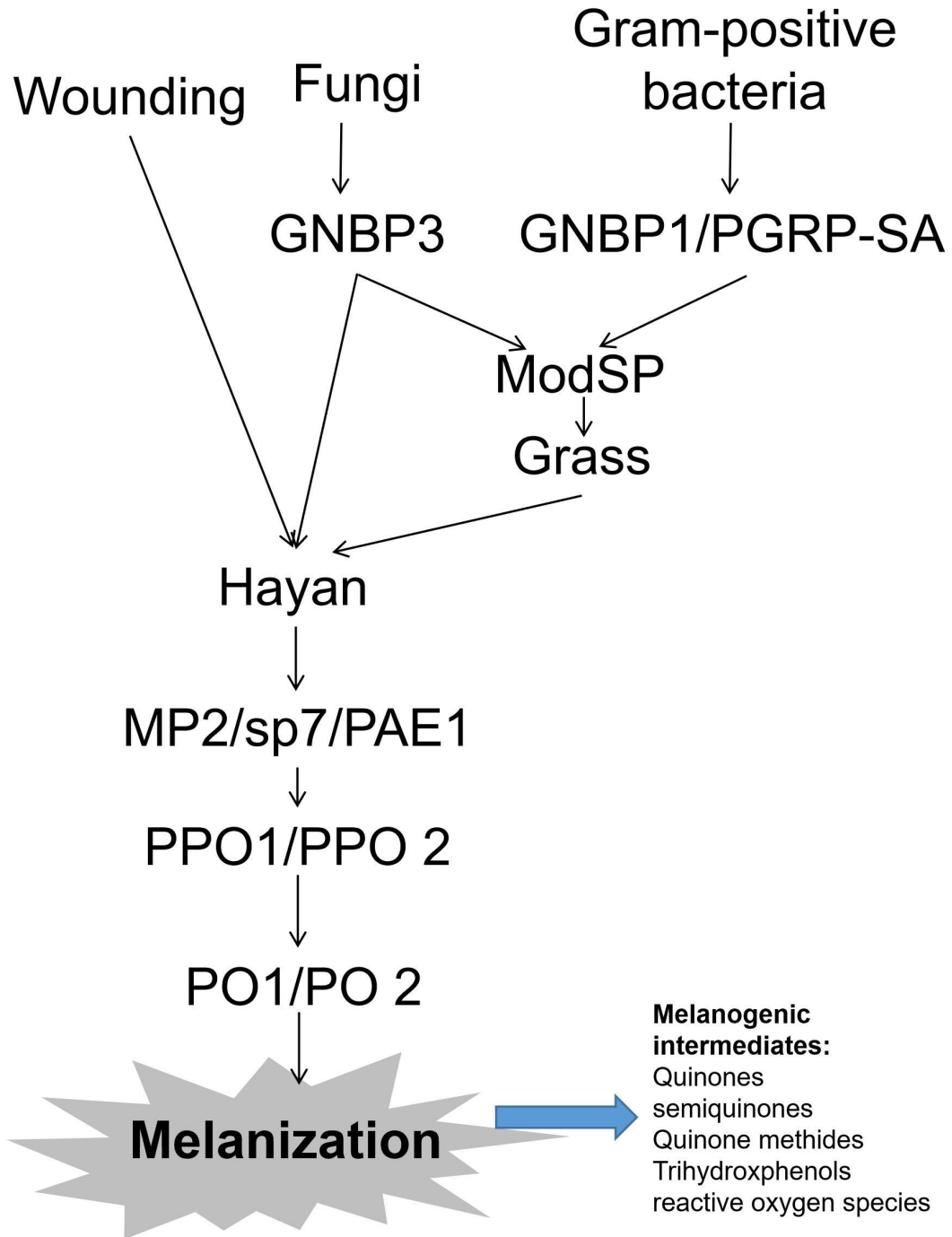


Figure 4: Overview of melanization. Physical injury active *Hayan* by proteolytic cleavage, that then to the pro-phenoloxidase 1 (PPO1) or PPO2 to initiate the process of melanization. Fungi and Gram-positive bacteria induce the expression of *Hayan* by pattern recognition receptors (GNBP3 for fungi, GNBP1/PGRP-SA for Gram-positive bacteria) via a part of the Toll pathway. Serine proteases cleave the PPOs into phenol oxidases (POs), POs are play the key role in synthesis of melanin. There are multiple byproducts made during the synthesis of melanin. Some of the melanogenic intermediates are cytotoxic and may eliminate the invading pathogens.

Fig 5

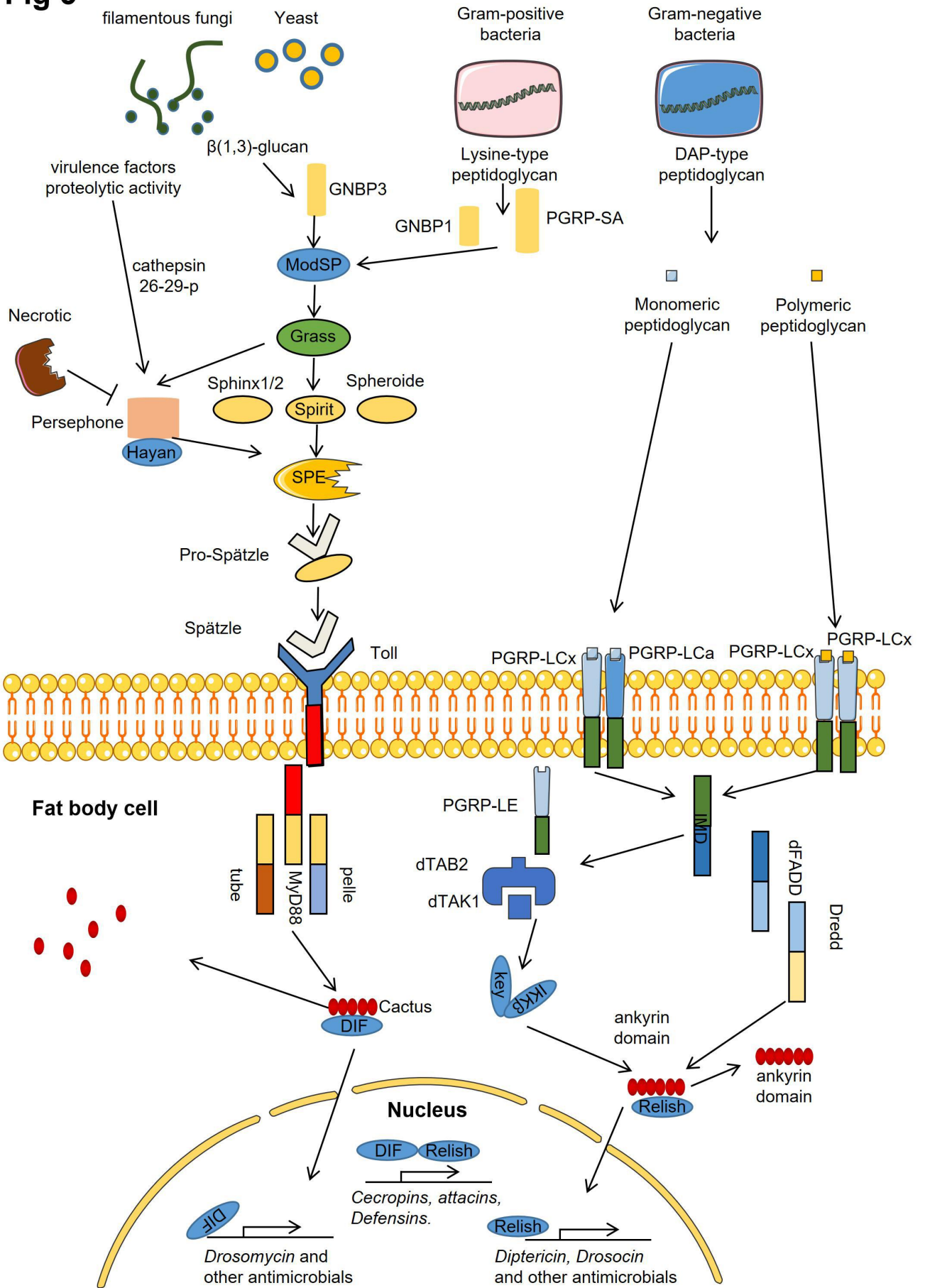


Figure 5: Model of Toll and IMD pathway activation. Different pathogens are detected by distinct pattern recognition receptors: the recognition of lysine-type peptidoglycan from Gram-positive bacteria mediated by GGBP1 and PGRP-SA, β -glucan from fungal cell walls is recognized by GGBP3. Persephone is proteolytically matured by virulence factors from fungi and bacteria, necrotic is a hemolymphatic serpin inhibit the activity of persephone. DAP-type peptidoglycan produced by Gram-negative bacteria is recognized by PGRP-LC, and PGRP-LE which is an intracellular receptor mainly exist in gut. Monomeric peptidoglycan is recognized by PGRP-LCx, PGRP-LCa and PGRP-LE, whereas polymeric peptidoglycan is recognized by PGRP-LCx. In Toll pathway, after Grass-SPE-Spätzle cascade initiated, serine protease cleaves proSpätzle into Spätzle. Spätzle is the ligand of Toll receptor. The signal is transduced within fat body cell by Toll receptor, then via MyD88, tube, pelle to active DIF to regulate the transcription of antimicrobial peptides. In IMD pathway, the NF- κ B Relish factor activated by two complementary ways: one is cleavage through the Dredd caspase thus liberating it from the ankyrin-repeat cytoplasmic anchor present in its C-terminal domain and thereby allowing nuclear uptake; the other phosphorylation of the N-terminal domain by the I- κ B Kinase complex that enables the transcription initiation transactivation properties of this transcription factor. In total, more than 230 genes regulated by Toll and IMD pathways. *DAP*, diaminopimelic acid; *GGBP*, Gram-negative bacteria binding protein; *PGRP*, peptidoglycan recognition protein; *Dredd*, Death related *ced-3/Nedd2-like caspase*.

**Chapter 1 *MyD88* plays an essential role in the
resilience to *Aspergillus fumigatus* infection in
Drosophila melanogaster but is not required to
prevent the dissemination of the fungus within
the fly**

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Abstract

Host defense encompasses two complementary dimensions, resistance, the immune response that results in the neutralization and killing of invading pathogens, and resilience/tolerance, the homeostatic reactions that participate in enduring and repairing damages inflicted either by pathogen's virulence factors or the host's own immune response.

Aspergillus fumigatus is a major human opportunistic pathogen that causes high morbidity and mortality in immunodeficient patients. As reported previously, *A. fumigatus* does kill Toll pathway immunodeficient *MyD88* flies but not wild-type flies. However, we observed that the fungal burden hardly increases in the mutant flies, even upon death, in contrast to other fungal infections. Some 250 injected conidia suffice to kill *MyD88* flies, in the absence of invasion of most fly tissues. In contrast, a mutant defective for melanization displays a reduced level of containment of the fungus, which then disseminates throughout the host body, yet is only weakly susceptible to *A. fumigatus* infection. Since *A. fumigatus* kills with a low fungal burden restricted to the injection site, we have therefore tested whether mycotoxins might be involved in pathogenesis and found that some of the many toxins known to be secreted by *A. fumigatus* differentially kill *MyD88* and not wild-type flies. We conclude that resilience to specific *A. fumigatus* mycotoxins and not the control of fungal dissemination appears to be the preponderant host defense against this infection. Future experiments will tell whether this host defense involves the Toll

pathway and whether it has also been conserved during evolution as has been the case for its function in resistance.

Introduction

Aspergillus fumigatus (*A. fumigatus*) is a rather ubiquitous saprotrophic fungus able to withstand high temperatures and to grow at 55°C (1). It thus is able to bypass the major barrier to fungal infections in warm-blooded animals: high body temperature. The dispersal form of *A. fumigatus* in the environment is the airborne conidium, the small size of which allows it to penetrate deeply in the airways of animals. Indeed, humans inhale daily hundreds to thousands of conidia (2), yet, mostly immunodeficient patients suffer from invasive aspergillosis (IA). Of note, some cases of IA affect apparently immunocompetent patients (3, 4). IA remains a major challenge to clinicians due to late diagnostic and high morbidity and mortality of this infectious disease.

A. fumigatus has a complex genome that allows it to adapt to varied environmental conditions and also to host defenses as well as to antifungal drugs. For instance, a range of efflux pumps and transporters protects it against toxic compounds, including possibly azoles used to cure IA (5). The virulence strategies of *A. fumigatus* are being deciphered and include intracellular germination of the ingested conidium that leads to mechanical lysis of the phagocyte (6). A characteristic of *Aspergillus* species is their rich secondary metabolism that allow them to produce a rich variety of metabolites, including mycotoxins. It has been hypothesized that the production of specific secondary metabolites by *A. fumigatus in vivo* contributes to its pathogenicity, particularly during hyphal growth. Gliotoxin (7), fumagillin (8), helvolic acid (9),

restrictocin (10), verruculogen (11), ergot alkaloids (12) and fumitremorgins (13) are mycotoxins secreted by *A. fumigatus*. Studies of gliotoxin revealed its immunosuppressive properties by inhibiting cellular effector functions or inducing cellular apoptosis (13-25). Up to now, gliotoxin is considered as the most important toxin produced by *A. fumigatus*. Fumagillin, which targets methionine aminopeptidase-2 (26, 27) has been used to treat corneal microsporidial *keratitis* (28) and has also been identified as an angiogenesis inhibitor (29, 30). Helvolic acid is a nortriterpenoid antibacterial compound that inhibits translation elongation factor 2 and oxidized low-density lipoprotein metabolism of bacteria (31, 32). Restrictocin is a protein produced by *Aspergilli* that belongs to the super family of ribonucleases and specifically cleave 28S ribosomal RNA at a specific loop (33, 34). These fungal ribotoxins are being developed into anticancer drugs (35). Verruculogen and fumitremorgin are prenylated indole alkaloids metabolites of *Aspergilli* which are tremorogenic. Their chemical structures are similar: fumitremorgin B can be converted into verruculogen by the FtmOx1 enzyme (36). Both act by reducing GABA levels in the central nervous system and as a result of reduced inhibition of the nervous system, tremors are induced (37, 38); they also inhibit the M phase of cell cycle to inhibit the cell proliferation (39). Ergot alkaloid are produced by many fungal species (40). One ergot alkaloid, festuclavine, interferes with several mammalian regulatory systems via its ability to bind to serotonin, to dopamine, and to α -adrenaline receptors (41). Although genes regulating the production of clavine ergot

alkaloids have been identified for *A. fumigatus* (42-44), the role of these metabolites in the pathogenesis of IA has yet to be explored.

Drosophila melanogaster is a genetic model organism that allows the exploration of many biological processes such as development, brain function, transcription or host defense against infections. Discoveries made in this model are often relevant to an understanding of our own biology; this may be linked to the relatively high degree of conservation during the evolution of animals, with up to 75% of genes known to cause disease in humans having a *Drosophila* homolog (45, 46). *Drosophila* host defense against systemic bacterial or fungal infections relies on both humoral and cellular arms (47). An invertebrate-specific host defense is melanization, which relies on the catalytic activity of phenol oxidase (PO) enzymes that are themselves activated by a proteolytic cleavage of their pro-forms. Active PO catalyzes the formation of melanin, for instance at wounds. A major protease required for proPO cleavage is Haya (48). The cellular immune response depends on hemocytes that carry on their surface potential phagocytic receptors such as Eater (49). The most studied antimicrobial responses are those mediated by potent antimicrobial peptides (AMPs), most of which have been identified through biochemical characterization of their activities. The expression of most AMPs is induced by immune challenges and regulated at the transcriptional level by two NF- κ B pathways, Immune deficiency (IMD) and Toll that function in the fat body, a tissue with mixed characteristics of hepatocytes and adipocytes, and hemocytes as well as barrier epithelia (IMD only) (50). The Toll pathway is essentially required for host defense against two strikingly

distinct types of microorganisms (51), Gram-positive bacteria harboring LYS-type peptidoglycan on their cell walls on the one hand, and fungi on the other, including *A. fumigatus*. The Toll pathway is activated by extracellular proteolytic cascades triggered either by the detection of $\beta(1-3)$ glucans or sensing of the proteolytic activity of fungal virulence factors (52). These proteolytic cascades converge and ultimately lead to the processing of Spätzle into an active Toll receptor ligand (53). Toll then activates the NF- κ B transcription factor Dorsal-related Immune Factor (DIF) through an intracellular pathway in which MyD88 plays a cardinal role (54). This pathway regulates the expression of tens of genes, the exact function in host defense of most of which remains to be delineated. A major readout of Toll pathway activation is *Drosomycin* mRNA expression. This gene encodes a potent AMP active against filamentous fungi. Other potential effectors of the Toll pathway include *Drosophila*-induced Immune Molecules (DIM)-encoding genes, first identified through mass-spectrometry analysis (55-57).

In this study, we characterize in detail the infectious process of *A. fumigatus* after the injection of a limited number of conidia into adult flies. Unexpectedly, we report that the fungus does not appear to grow to high levels, even in immunodeficient *MyD88* mutants that nevertheless succumb to the infection. Our investigations reveal that the effective host defense against *A. fumigatus* does not consist in limiting the dissemination of the fungus within the host but rather depends on being able to withstand or neutralize the action of mycotoxins, a process belonging to resilience (also known as tolerance) rather than resistance.

Results

Characterization of A. fumigatus in wild-type and MyD88 flies

As we have not been able to infect flies by incubating them in contact with conidia or by feeding them conidia (58), we have resorted to the classical injection model and first performed a dose-response analysis in both wild-type and *MyD88* immunodeficient flies (**Fig. 1A, Fig. S1A**). As reported previously, wild-type flies were not killed by any dose of injected conidia, up to 500 conidia per fly. In contrast, as few as five injected conidia per fly were sufficient to efficiently kill *MyD88* flies within a week. We also tested several different wild-type *A. fumigatus* strains and obtained similar results for a dose of 250 injected conidia. Next, we examined the development of a GFP-labeled fungus within its host. The small size of conidia yields a low-intensity signal that makes them difficult to detect and we managed to observe a handful of swollen, maturing conidia in either wild-type or *MyD88* flies. In contrast, hyphae were detected next to the injection site by 20 hours after infection in about half of the flies (**Fig.1, B-D**). We did not detect any difference of behavior of *A. fumigatus* between wild-type or mutant flies. We next monitored the fungal burden on single flies 24 and 48 hours post-infection. Strikingly, the number of colony-forming units (CFUs) did not increase after injection in wild-type or *MyD88* flies, in contrast to other pathogens controlled by the Toll pathway (**Fig. 1E, F**). This was observed for two injected doses, 250 and 5000 conidia. To exclude the possibility of a late proliferation of the fungus occurring just prior to the fly demise, we performed a

Fungal Load Upon Death (FLUD) analysis on single flies and found that the number of colony-forming units did not increase much: about 200 CFUs whether we injected a low or a higher dose of conidia (**Fig. 1G**). Next, we monitored the expression of *Drosomycin* as read-outs of Toll pathway activation. Only the injection of 500 live conidia yielded a strong induction of *Drosomycin* that was *MyD88*-dependent (**Fig. 1H**). UV-killed conidia were hardly inducing *Drosomycin* expression, suggesting that the β -(1-3)-glucan of the fungal cell wall is well hidden, possibly by hydrophobins. In conclusion, in contrast to most infections studied to date in *Drosophila*, a limited number of Colony-Forming Units appears to be sufficient to kill *MyD88* flies.

Hayan appears to mediate a host defense that limit the dissemination of the fungus within the fly

As *MyD88*'s role in controlling the proliferation of injected *A. fumigatus* is limited at best, we investigated other host defenses. We first tested the IMD pathway component *Kenny*, which encodes fly NEMO/IKK γ . Indeed, *Relish* has been reported to be sensitive to some fungal infections. We found that *key* flies were somewhat susceptible to injected *A. fumigatus* (**Fig. 2A**). Of note, we never observed any fungus emerging from cadavers, in contrast to *MyD88* mutants. Our attempts to measure the FLUD on those flies revealed only a few CFUs (**Fig. 2B**), suggesting that flies did not directly succumb to fungal infections or at least to uncontrolled fungal proliferation.

Next, we tested a possible contribution to the host defense against *A. fumigatus* of the cellular immune response. To this end, we tested two mutant combinations in which the expression of the putative phagocytic receptor Eater is ablated and found no

increased susceptibility to infection (**Fig. 2C**). We then saturated the phagocytic apparatus by the prior injection of nondegradable polystyrene beads and obtained similar results (**Fig. 2D**). Finally, we generated flies in which hemocytes are killed during development by the expression of the pro-apoptotic genes *reaper* and *head involution-defective* (59, 60). These flies were as resistant as wild-type flies to the *A. fumigatus* challenge (**Fig. 2E**).

The Hayan protease is required for the activation of pro-phenol oxidases into mature, active phenol oxidases that catalyze multiple steps that lead to the deposition of melanin at the wound site and also possibly on the pathogens. The enzymatic reactions are also thought to release Reactive Oxygen Species. *Hayan* mutant flies displayed a moderate susceptibility to injected *A. fumigatus* conidia (**Fig. 2F**). Unexpectedly, we found that *Hayan* flies displayed a much-enhanced fungal load upon death as compared to *MyD88* flies (**Fig. 2G**). *PPO2* mutant flies displayed a survival similar to that of *MyD88* flies after *A. fumigatus* infection (**Fig. 2H**). This finding may mirror a requirement for another protease besides Hayan to activate PPO2. We noted that whereas *A. fumigatus* mycelium developed only on the thorax of *MyD88* cadavers as reported previously, most of the body of the deceased *Hayan* flies was covered by the fungus (**Fig. 2I-J**). We corroborated this finding by observing the development of the fungus during infection using a GFP-labeled strain. In contrast to *MyD88* flies, hyphae observation was not limited to the thorax and extended to both head and abdomen (**Fig. S2**). We conclude that *Hayan* is required to limit the dissemination of *A. fumigatus* away from the wound site.

MyD88 is required for resilience to A. fumigatus toxins

A. fumigatus is known to produce an armamentarium of toxins such as gliotoxin and fumagillin. We first tested an *A. fumigatus* mutant devoid of any secondary metabolism, *ApptA* and found this mutant to be avirulent (**Fig. 3A**), even though we were able to detect hyphae next to the wound site (**Fig. S3**). The fungal burden was however reduced for the mutant as compared to the virulent strain (**Fig. 3B**). Gliotoxin has been reported to be a virulence factor of *A. fumigatus* both in mice and flies. Under our conditions, however, a Δ gliP strain affecting the non-ribosomal peptide synthetase that catalyzes the first step of the gliotoxin biosynthesis pathway displayed a wild-type virulence (**Fig. S4A**). We also injected the gliotoxin at various concentrations and were unable to find a concentration that would preferentially kill *MyD88* flies (**Fig. S4B**). The injection of fumagillin or helvolic acid did not kill wild-type or *MyD88* flies at the tested concentrations (**Fig. S5**). In contrast, the injection of restrictocin at three concentrations only killed *MyD88* flies (**Fig. 3C**). The injection of a low concentration (0.1 mg/ml) of verruculogen did not kill *MyD88* or wild-type flies. Interestingly, verruculogen at higher concentrations (1 or 5 mg/ml) killed *MyD88* flies faster than wild-type flies whereas a 10 mg/ml concentration killed both fly strains within a day (**Fig. 3D**). An ergot alkaloid derivative killed only *MyD88* flies when delivered at a concentration of 1 or 10 mg/ml whereas a dose of 150 mg/ml was lethal to both (**Fig. 3E**). Finally, fumitremorgin C at 1 mg/ml killed only *MyD88* flies (**Fig. 3F**). These experiments had been performed at 29°C. When we repeated them at 18°C, the effect was still present, although it was delayed, in

keeping with similar results obtained after the injection of *A. fumigatus* conidia (**Fig. S6**). As we sometimes found a few bacteria in the hemolymph of injected flies (**Fig. S7**), to ensure that immuno-deficient *MyD88* flies were truly killed by the toxins and not by opportunistic infections triggered by toxin injection, originating for instance from the microbiota, we repeated these experiments on antibiotics-treated or axenic flies and obtained similar results (**Fig. S8**). Thus, *MyD88* flies succumb to the effect of the toxin, even in the absence of the microbiota.

To determine whether the injection of toxins may activate the Toll pathway, we monitored the expression of *Drosomycin* (**Fig. 3G**). There was no significant induction by any of the toxins.

Discussion

The function of the *Drosophila* Toll pathway in host defense was first revealed in a landmark study in which the susceptibility of several Toll pathway mutants to *A. fumigatus* infection was correlated to an impaired expression of *Drosomycin* in these mutants, an observation in keeping with the antifungal activity of *Drosomycin* on hyphae (61, 62). Further genetic investigations showed that the overexpression of *Drosomycin* in a *Spätzle* mutant background provided limited but nevertheless significant protection against *A. fumigatus* (63). A recent study has also reported a mild sensitivity of flies devoid of most AMP-encoding genes, including *Drosomycin* and *Metchnikowin* (64). The inoculation load in these studies is difficult to assess as it relied on pricking flies with a needle previously dipped into a concentrated conidial solution. An open possibility is that the dose used in these studies may have been much higher than those in our work, possibly close to 20,000 conidia (58). It is possible that higher doses of the fungi may reveal a fungicidal or fungistatic action of host defenses regulated by the Toll pathway mediated by *Drosomycin*, *Metchnikowin*, and/or DIMs. The fungal load has not been determined in neither of these previous studies. In contrast, using a “rolling” assay, Lionakis *et al.* reported an increased fungal burden in Toll pathway mutants infected in the “rolling” assay (58). They nevertheless did not comment their data showing not an increasing but a decreasing fungal load as the infection proceeded, in both wild-type and Toll mutants. Under our low inoculum conditions, the Toll pathway does not appear to be required to limit the

proliferation of the invading fungus. Indeed, a dose of 50 injected conidia suffices to kill *MyD88* flies yet does not trigger a detectable activation of the Toll pathway in wild-type flies. In contrast, melanization appears to be important to prevent the dissemination of the fungus, a finding in keeping with a recent study documenting a similar role in host defense against a low inoculum of *S. aureus* (65). Strikingly, *Hayan* melanization-deficient flies, even though they harbor a higher fungal load upon death, are nevertheless much more resistant to *A. fumigatus* than *MyD88* flies. It follows that the control of the dissemination of the fungus is not a critical parameter of the host defense against this infection, even though it is often the case, for instance with *Candida glabrata* or *Enterococcus faecalis*.

Rather, our data suggest that the ability to cope with the exposure to mycotoxin is the relevant host defense present in wild-type and *hayan* flies, but lacking in *MyD88* flies, even when the Toll pathway does not appear to be stimulated in the case of a low inoculum dose. Furthermore, the injection of toxins did not trigger an increased expression of *Drosomycin*. Our finding that an *A. fumigatus* strain devoid of secondary metabolism is less virulent supports this hypothesis, even though the restrictocin protein is still expected to be produced in this strain. Of note, many toxins appear to be produced predominantly by hyphae and not conidia (66); hyphae appear to be formed at the site of injection of conidia and our data suggest that a limited quantity of hyphae is sufficient to kill the flies in the absence of the invasion of tissues. Thus, we envision that they are able to release enough mycotoxins to kill the flies. Indeed, some fungal mycotoxins target the nervous system; for instance, verruculogen

decreases the levels of the major inhibitory mediator of neural activity, GABA. Altering GABA levels may lead to uncontrolled behavior and seizures. Within six hours of the injection of verruculogen, 60-80% of the flies cannot keep their gait and balance and fall down on the fly food, unable to stand and walk, yet frantically moving their legs in the air. Although their sports ability seems did not impaired, their legs struggling vehemently, but none of them can stand or run. This behavior lasts between 24 to 72 hours until the ultimate demise of the flies. The same behavior, including its duration, is observed in 20 to 50% of flies infected by *A. fumigatus*, suggesting that indeed some neurotoxins affect the brains of a significant fraction of the hosts. Future studies will determine whether different toxins synergize in killing the host. To this end, it will be important to generate fungal strains unable to produce subsets of toxins. Nevertheless, the injection of mycotoxins establishes that *MyD88* flies are more sensitive than wild-type to some but not all *A. fumigatus* secreted mycotoxins.

At this stage, it is not clear whether the sensitivity to some mycotoxins is shared by mutants affecting other components of the Toll pathway. As most of these Toll pathway mutants (*Spätzle*, *Toll*, *Tube*, *pelle*) were sensitive to *A. fumigatus* infections (62), at least to a high dose, it is likely that these mutants will also display a sensitivity to these mycotoxins. It will also be interesting to determine whether flies harboring a deficiency that removes a cluster of ten DIM genes and that phenocopies the Toll pathway infection sensitivity phenotype are also sensitive to the same set of mycotoxins (55). At present, however, we cannot formally exclude that *MyD88* itself

is not causing this effect, which might be due to a second site mutation on the chromosome, a possibility being currently tested. Even if this were the case, our present data clearly establish that the important phenotype of *MyD88* flies is not a defective immune response but an impaired resilience to mycotoxins. As *MyD88* flies are sensitive to very low doses of *A. fumigatus* that do not trigger the Toll pathway, it is likely that the resilience function is constitutive and not inducible by infection, unless wounding in itself does trigger a response that is distinct from the AMP one. It will be important to determine whether *MyD88* flies have impaired detoxification functions, which in flies are predominantly regulated by the Nrf2 and HR96 transcription factors. Systematic unbiased genetic analysis will provide further clues to decipher how *Drosophila* is able to prevent, elude or neutralize mycotoxins. Hopefully, these mechanisms have been conserved throughout evolution and our current work may lead to a better understanding as to how mammals and humans cope with these mycotoxins. An open possibility is that some immunocompetent patients suffering from IA may actually have a defective resilience to mycotoxin action.

Material and methods

Pathogens culture. *Aspergillus fumigatus* was cultured on potato dextrose agar (PDA) medium + 0.1g/l chloramphenicol (Huankai Microbio Tech) in a tissue culture incubator under 5% CO₂ at 29 °C. Conidia were harvested at 4-7 days of the culturing. The conidial suspension was purified by filtration on cheese cloth to eliminate hyphae and other impurities. Our standard wild-type *Aspergillus fumigatus* (we used Af as its symbol in our research) is a kind gift from Drs. Anne Beauvais and Jean-Paul Latge (Institut Pasteur, Paris), the other wild-type strains which include D141 (background of D141-GFP), Af293, ATCC46645, *CEA17ΔakuB^{Ku80}* (background of *ΔgliP*), A1160 (background of *ΔpptA*), GFP labeled strain (D141-GFP) and *ΔpptA* (secondary metabolites free mutant) (67), *ΔgliP* (gliotoxin free mutant) (6) mutants *Aspergillus fumigatus* are kind gifts from Dr. Axel Brakhage (Friedrich-Schiller-Universität Jena).

Micrococcus luteus (*M. luteus*) was cultured in Tryptic soy broth (TSB) at 37 °C for 24h, and centrifuged at 3000 round per minute for 10 minutes, after that we discarded supernatant, and resuspended *M. luteus* with 1 ml PBS. Repeated this protocol twice to eliminate medium and then measured OD value by spectrophotometer (Amersham Biosciences).

Toxins preparation. Restrictocin (Sigma) was resuspended in phosphate buffer saline (PBS) pH = 7.2, gliotoxin (Sigma), helvolic acid (Sigma), fumagillin (Sigma), verruculogen (Abcom), fumitremorgin C (Sigma), bromocriptine mesylate (Sigma)

were dissolved in pure Dimethyl sulfoxide (DMSO) (Sigma) at a concentration of 10 mg/ml for restrictocin, gliotoxin, helvolic acid, fumagillin, and verruculogen, 1 mg/ml fumitremorgin C, 150 mg/ml bromocriptine mesylate as the stock to store in -20°C. Working concentrations in pure DMSO (1 mg/ml restrictocin, 1 mg/ml verruculogen, 1 mg/ml fumitremorgin C, 30 mg/ml bromocriptine mesylate) were stored at -20°C. The working concentrations were used for injection unless otherwise indicated. They were thawed at room temperature for one hour prior to use. As multiple freeze/thaw cycles reduce the virulence of the toxins, care was taken not to use an aliquot more than five times and aliquots were not stored for more than a month.

Fly strains. Fly lines were raised on media at 25 °C with 65% humidity. For 25 l of fly food medium, 1.2 kg cornmeal (Priméal), 1.2 kg glucose (Tereos Syral), 1.5 kg yeast (Bio Springer), 90 g nipagin (VWR Chemicals) diluted into 350 ml ethanol (Sigma-Aldrich), 120 g agar-agar (Sobigel) and water qsp were used.

w⁴⁵⁰⁰¹ flies were used as wild-type control and the mutants *MyD88^{-/-}*, *Hayan^{-/-}*, *key^{-/-}* are all from Exelixis (68), *eater1* is a kind gift from Dr. Bruno Lemaitre (69), *eaterΔ* is crossed by two mutant lines *Df(3R)T1-I*, *e¹/TM3*, *Ser¹* (BDSC1911) and *Df(3R)D605/TM3*, *Sb¹ Ser¹* (BDSC823) which from Bloomington Drosophila Stock Center in our lab. Canton-S (BDSC64349), *w¹¹¹⁸* (VDRC60000), *y¹w¹* were used as wild-type controls.

hmlΔ Gal4 UAS-eGFP is a reported line (70), *UAS-rpr-UAS-hid* flies is crossed by a reported line *w;;P[UAS-hid]* with *w¹¹¹⁸*; *P{UAS-rpr.C}14* (BDSC5824) which from

Bloomington *Drosophila* Stock Center in our lab (59, 60). Were crossed and hatched in 29 °C.

To obtain axenic flies, eggs were collected, washed with water and then 70% ethanol prior to dechoriation by pipetting up and down eggs in a solution of 50% bleach until the chorion disappeared. Eggs were transferred into sterile vials containing media and a mix of antibiotics: ampicillin, chloramphenicol, erythromycin and tetracycline. Once emerged, adult flies were crushed and tested on LB-, bacitracin-heated blood-, MRS and yeast peptone dextrose-agar plate to observe any contamination by bacteria, fungi or yeast. Of note, no anaerobic microorganisms have been detected in the *Drosophila* microbiota.

Flies were treated with antibiotics mix, which contain Ampicillin, Tetracycline, Chloramphenicol, Erythromycin, Kanamycin. The antibiotics mix was added into fly food which the final concentration is 50 µg/ml for each. Females were collected after two generations cultured on the fly food with antibiotics. The micro-biotic test is same to axenic flies.

***Aspergillus fumigatus* infection.** For *Aspergillus fumigatus* infection, spores were prepared freshly for each infection. Unless otherwise stated, spores were injected into the thorax, precisely into the mesopleuron on adult flies at a concentration of 250 spores in 4.6 nl PBS containing 0.01% Tween20 (PBST) using a microcapillary connected to a Nanoject II Auto-Nanoliter Injector (Drummond). The same volume of PBS-0.01% Tween20 was injected for control experiments. All experiments were performed at 29 °C unless otherwise indicated. Before all the experiments, the flies

were put in tubes with only 100 mM sucrose solution to do a 48 hours amino-starvation unless otherwise indicated.

Toxins injection. Toxins injection were performed as for *A. fumigatus* injection, except that a toxin solution was used instead of a spore suspension.

Saturation of phagocytosis. Latex beads treatment was performed as previously described (51). We put the treated flies on 100 mM sucrose solution for 48 hours and then do the injection.

Survival tests. Survival tests were performed using 20 flies per vial in biological triplicates. Adult flies used for survival tests were 5–7-days old from 25 °C stock. For survival tests using RNAi-silencing genes, flies were kept for 5 more days at 29 °C to allow the expression of the RNAi transgene prior to the experiment. Flies were counted every day. Each experiment shown is representative of at least three independent experiments.

Fungus quantification. Fungus quantification was determined using single adult flies per condition. Flies were transferred into multi-tubes (Starstedt) containing two 1.4-mm ceramic beads (Dominique Dutcher) in 100 µl PBS-0.01% Tween20. Single flies was smashed by shaker (F. Kurt Retsch GmbH & Co. KG) with 30/min, 30 seconds for twice. Then we plated the smashed suspension on potato dextrose agar (PDA) + antibiotic plates. After that, the plates were enclosed with parafilm and cultured at 29 °C with 65% humidity, after 48 h to count the colonies by eyes. FLUD was performed as described (71).

***In vivo* checking of conidial development.** Flies were sacrificed and dissected in 8-well diagnostic microscope slides (Thermo Scientific) and analyzed under Zeiss stereomicroscope microscope (Carl Zeiss). D141 UVITEX was used for negative staining of $\Delta pptA$'s hyphae, by adding to each well 5 μ l UVITEX for 30 seconds at room temperature. Flies injected by D141-GFP and $\Delta pptA$ were dissected and observed under a fluorescent Zeiss axioscope microscope (Carl Zeiss) each hour after the injection.

Hemolymph extraction. Hemolymph was extracted by microcapillary connected to a Nanoject II Auto-Nanoliter Injector (Drummond) to injected into the thorax, precisely into the mesopleuron on adult flies. The hemolymph from single fly or 5 flies was expelled into 100 μ l PBS-0.01% Tween20 and mixed gently by pipettes. We plated 50 μ l of the hemolymph suspension on LB-yeast peptone dextrose-agar plates. The plates were put in a tissue culture incubator under 5% CO₂ at 29 °C for 48h and count the bacterial single colony by eyes.

UV-killed pathogens preparation. *A. fumigatus*: The conidial suspension was plated on potato dextrose agar (PDA) + 0.1g/l chloramphenicol plates, exposed to the UV-light after the plates dry for 3 h twice. Enclosed plates with parafilm and cultured at 29 °C with 65% humidity, after 48 h to check the colonies. Sort the plates without any colony, resuspend the dead conidia to measure the concentration and then do the injection.

M. luteus: bacterial suspension was plated on Tryptic soy broth (TSB) plates, exposed to the UV-light after the plates dry for 3 h. The rest protocols are same to the *A. fumigatus*.

Scanning electron microscope. Treat flies in the mixture as follows: 500 μ L phosphate buffer 0,2M pH 7.2, 250 μ L ddH₂O, 100 μ L glutaraldehyde 25% (2.5% final), 150 μ L paraformaldehyde 16% (2.4% final) in room temperature for more than one hour. Then fix flies with resin and do the observation by SEM (ZEISS).

Drosomycin expression measurement. Expression of Drosomycin was measured by RT-qPCR as as described previously (62).

Statistical analysis and reproducibility. All statistical analyses were performed using Prism 7 (GraphPad Software). The Mann-Whitney and/or Kruskal-Wallis tests were used unless otherwise indicated. For survival experiments, we use log-rank test. When using parametric tests (analysis of variance (ANOVA) and t-test), a Gaussian distribution of data was checked using either D'Agostino-Pearson omnibus or Shapiro-Wilk normality tests. All experiments were performed at least three times. Significance values: *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

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Figure legends

Figure 1: *A. fumigatus* does not proliferate in *MyD88* mutants.

(A) Survival of *MyD88* flies exposed to several doses of injected *A. fumigatus* conidia. There was no significant difference in survival between control flies injected with PBST (Phosphate buffer saline containing 0.01% Tween20) and flies injected with 1 conidium on average per fly (c/f) ($P = 0.9230$). All other injected doses of *A. fumigatus* led to the demise of infected flies as compared to control flies ($P < 0.0001$).

(B-D) Flies were injected with conidia of the D141-GFP *A. fumigatus* strain and hyphae (arrows) directly observed at the injection point with a fluorescence microscope 24 hours afterwards. (B-B'): wild-type (*wt*) flies injected with 500 conidia; B: bright field, B': fluorescence observed using a set of GFP filters. (C-D) respectively low (50 conidia) and high (500 conidia) doses injected into *MyD88* flies. Note that more hyphae were repeatedly detected using the low dose. The fungus was detected only at the injection point in all conditions.

(E-F) Fungal burden measured at the indicated time points using a plating assay (cfu: colony forming unit) of single *wt* or *MyD88* flies after the injection of a low (250 conidia) (E) or a high (5000 conidia) *A. fumigatus* inoculum. NS: not significant. *****: ($P < 0.0001$).

(G) Fungal load upon death of single flies injected with the indicated doses of *A. fumigatus* conidia (1×10^7 = 50 conidia per fly, 5×10^7 = 250 conidia per fly, and 10^8 = 500 conidia per fly). ***: ($P = 0.0002$), *****: ($P < 0.0001$).

(H) *Drosomycin* expression levels as measured by RTqPCR after the injection of either live or killed *A. fumigatus* at different concentrations (10 [7] = 50 conidia per fly, 10 [8] = 500 conidia per fly). PBST-injected flies were used as a negative control, and all the data were normalized to the *M. luteus* injected group set at a value of 100. The *Drosomycin* expression levels of *wt* after the injection of 500 conidia are similar to those induced by the injection of *M. luteus* ($P = 0.9314$); in contrast, all other groups do not display a significant induction of *Drosomycin* expression ($P < 0.0001$), including the injection of 50 live conidia.

All the survival data were analyzed using the log-rank test, the fungal burden and *Drosomycin* expression level data were analyzed using the Kruskal-Wallis with post-hoc tests to compare specific pairs. All experiments have been performed at least three independent times.

Figure 2: Hyan, and not other host defenses, limits the dissemination of *A. fumigatus* within the fly.

(A) Survival of *key* flies exposed to 500 conidia per fly of injected *A. fumigatus*. *key* flies after the injection of *A. fumigatus* are died faster than control flies injected with PBST (Phosphate buffer saline containing 0.01% Tween20) ($P < 0.0001$).

(B) Fungal burden measured at the indicated time points using a plating assay (cfu: colony forming unit) of single *key* or *MyD88* flies after the injection of 500 conidia of *A. fumigatus*. NS: not significant. ****: ($P < 0.0001$).

(C-E) Survival of cellular response deficient flies exposed to 500 conidia per fly of injected *A. fumigatus*. (C) There was no significant difference in survival between control wild-type (*wt*) flies and *eater* mutant flies *eater¹* and *eat^Δ* injected with *A. fumigatus* ($P = 0.9990$). (D) There was no significant difference in survival of latex beads saturated *wt* flies between control injected with PBST and injected with *A. fumigatus* ($P = 0.9992$). (E) There was no significant difference in survival between control *wt* flies and phago-hemoless UAS-rpr-UAS-Hid flies injected with *A. fumigatus* ($P = 0.9984$).

(F) Survival of *Hayan* flies exposed to 500 conidia per fly of injected *A. fumigatus*. *Hayan* flies after the injection of *A. fumigatus* are died faster than control flies injected with PBST ($P < 0.0001$), but died slower than *MyD88* injected with *A. fumigatus* ($P < 0.0001$).

(G) Fungal load upon death of single flies injected with 500 conidia per fly of *A. fumigatus*. NS: not significant. ****: ($P < 0.0001$). D141-GFP was used in this test, it have the similar virulence with our standard *A. fumigatus*, but less colonies when plating.

(H) Survival of *PPO2* flies exposed to 500 conidia per fly of injected *A. fumigatus*. *PPO2* flies after the injection of *A. fumigatus* are died faster than control flies injected with PBST ($P < 0.0001$).

(I) Hyphae on the surface of bodies of *MyD88* flies are only exist on the thorax (arrow).

(J) Hyphae on the surface of bodies of *Hayan* flies can be observed on the head (upper right arrow), thorax (upper left arrow), abdomen (lower left arrow) and legs (lower right arrow).

All the survival data were analyzed using the log-rank test, the fungal burden data were analyzed using the Kruskal-Wallis with post-hoc tests to compare specific pairs.

All experiments have been performed at least three independent times.

Fig. 3: MyD88 is sensitive to some *A. fumigatus* toxins.

(A) Survival of *MyD88* flies exposed to 500 conidia per fly of injected Δ *pptA* (secondary metabolites free mutant) *A. fumigatus*. *MyD88* flies after the injection of Δ *pptA* *A. fumigatus* are died slower than control flies injected with wild-type (*wt*) *A. fumigatus* ($P < 0.0001$).

(B) Fungal burden measured at the indicated time points using a plating assay (cfu: colony forming unit) of single *MyD88* or *wt* flies after the injection of 500 conidia of Δ *pptA* *A. fumigatus*. ****: ($P = 0.0001$), ****: ($P < 0.0001$).

(C-F) Survival of *MyD88* and *wt* flies exposed to several doses of injected mycotoxins. (C) *MyD88* flies after injected with indicated doses of restrictocin (R) (1 mg/ml = 5 ng per fly, 5 mg/ml = 25 ng per fly, 10 mg/ml = 50 ng per fly) died faster than the *wt* flies injected with restrictocin ($P < 0.0001$), and the control *MyD88* flies injected with PBST (Phosphate buffer saline containing 0.01% Tween20) ($P < 0.0001$). (D) *MyD88* flies after injected with 1 or 5 mg/ml verruculogen (V) died faster than the *wt* flies injected with verruculogen ($P < 0.0001$), and the control

MyD88 flies injected DMSO (Dimethyl sulfoxide) ($P < 0.0001$). There was no significant difference in survival between control *MyD88* flies injected with DMSO and *MyD88* flies injected with 0.1 ($P = 0.6368$) or 10 ($P = 1$) mg/ml of verruculogen (0.1 mg/ml = 0.5 ng per fly). (E) *MyD88* flies after injected with 5 or 10 mg/ml Bromocriptine mesylate (B) died faster than the *wt* flies injected with Bromocriptine mesylate ($P < 0.0001$), and the control *MyD88* flies injected DMSO ($P < 0.0001$). There was no significant difference in survival between *MyD88* flies injected with DMSO and *MyD88* flies injected with 150 ($P = 1$) mg/ml of Bromocriptine mesylate (150 mg/ml = 750 ng per fly). (F) *MyD88* flies after injected with 1 mg/ml fumitremorgin C (FC) died faster than the *wt* flies injected with fumitremorgin C ($P < 0.0001$), and the control *MyD88* flies injected DMSO ($P < 0.0001$).

(G) *Drosomycin* expression levels as measured by RTqPCR after the injection of restrictocin (R, 5 ng per fly), verruculogen (V, 5 ng per fly), Bromocriptine mesylate (B, 150 ng per fly), fumitremorgin C (FC, 5 ng per fly). PBST or DMSO -injected flies were used as negative controls, and all the data were normalized to the *M. luteus* injected group set at a value of 100. The *Drosomycin* expression level of *wt* after the injection of restrictocin is similar to those induced by the injection of PBST ($P = 0.9314$). The *Drosomycin* expression levels of *wt* after the injection of verruculogen ($P = 0.1615$), Bromocriptine mesylate ($P = 0.1672$), fumitremorgin C ($P = 0.2224$) are similar to those induced by the injection of DMSO.

All the survival data were analyzed using the log-rank test, the fungal burden and *Drosomycin* expression level data were analyzed using the Kruskal-Wallis with

post-hoc tests to compare specific pairs. All experiments have been performed at least three independent times.

Figures

Figure 1

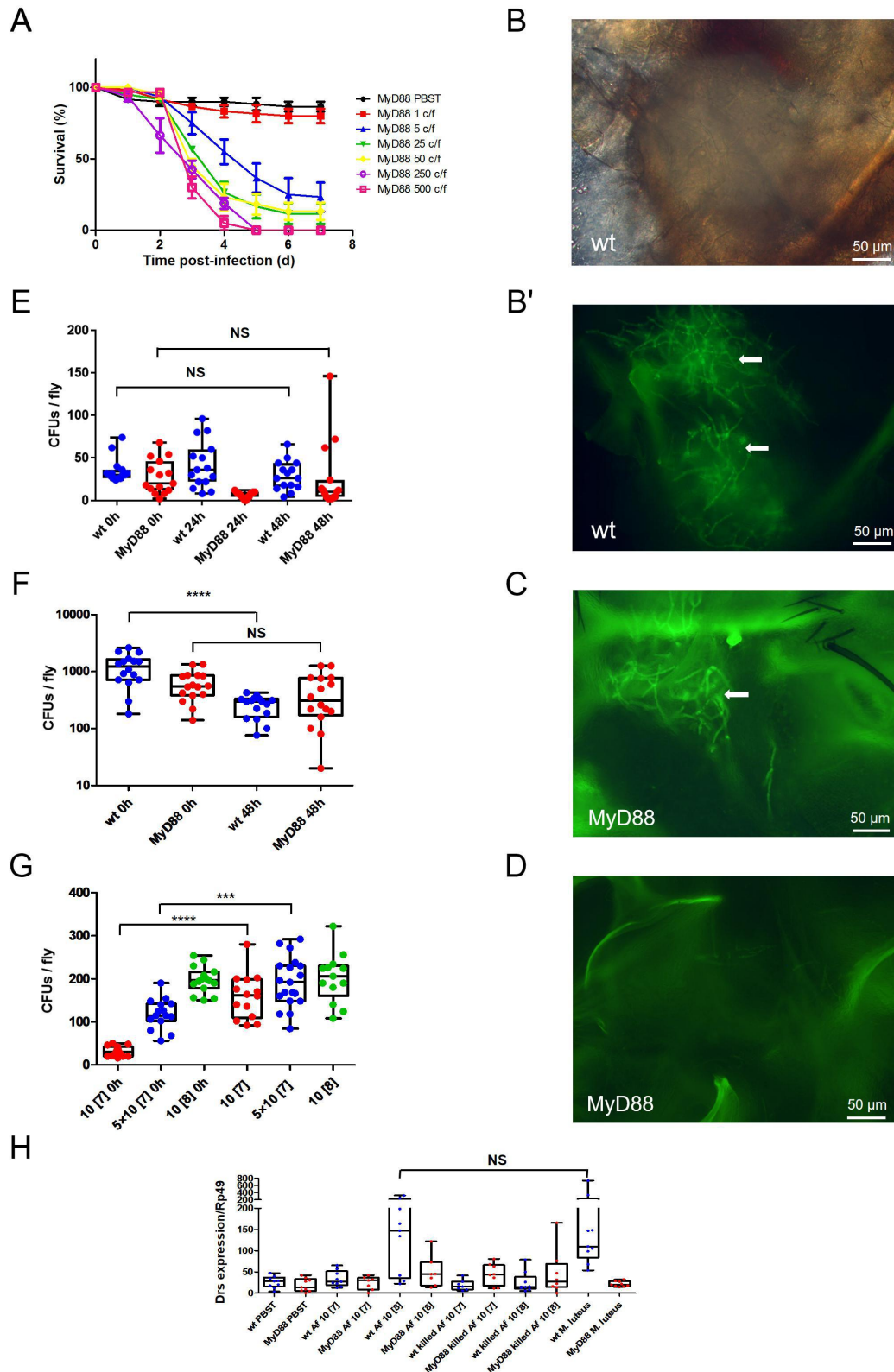


Figure 2

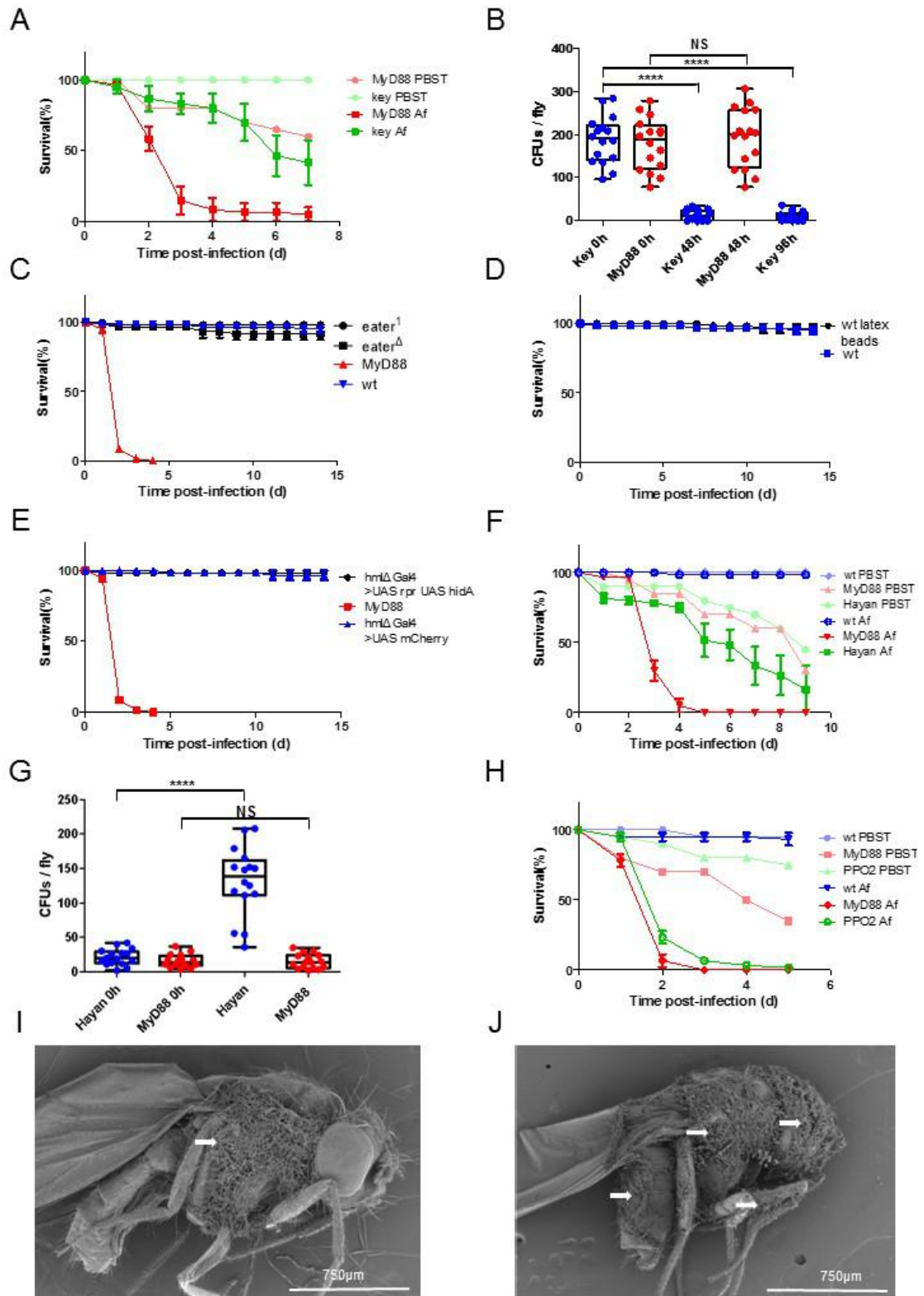
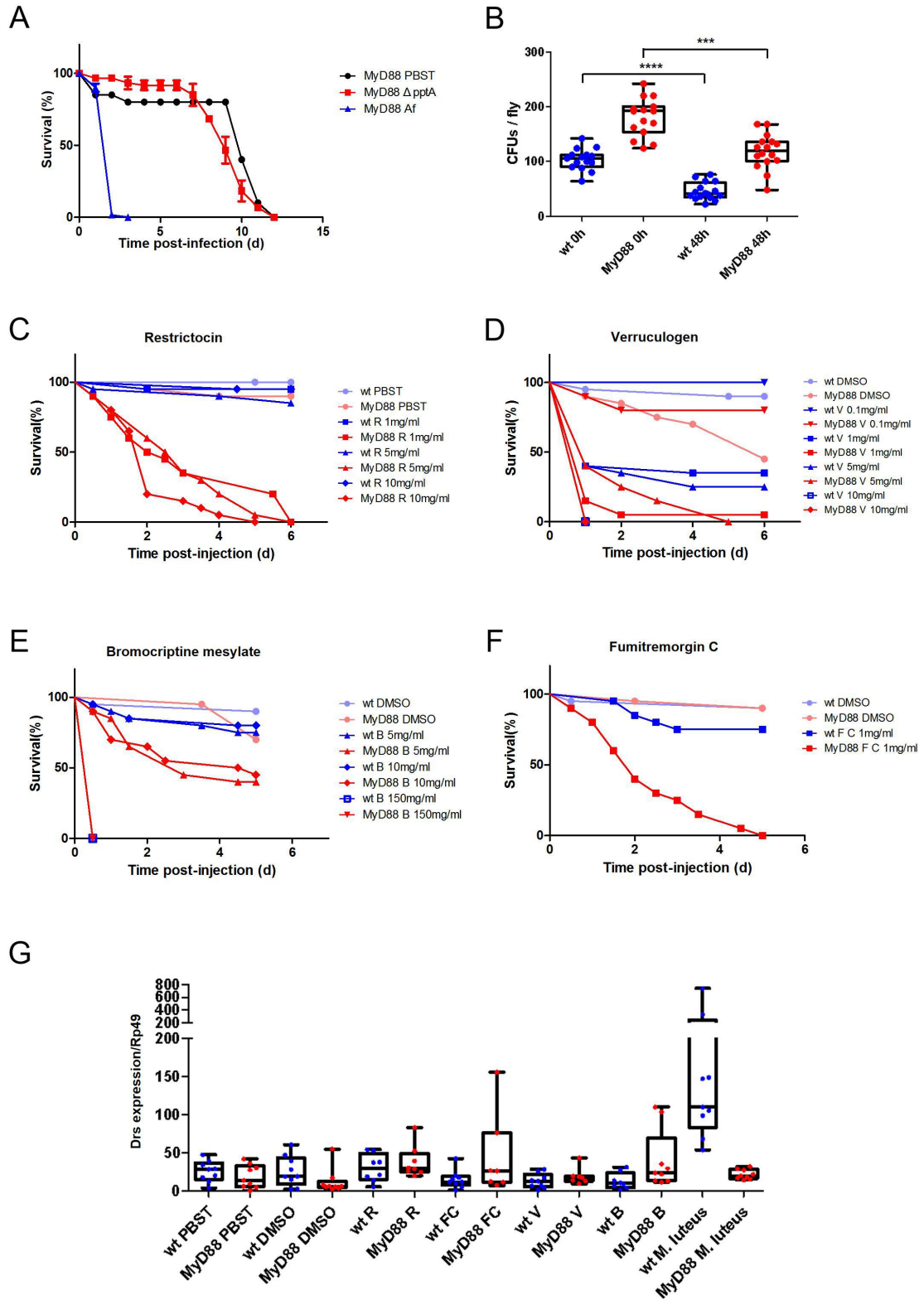


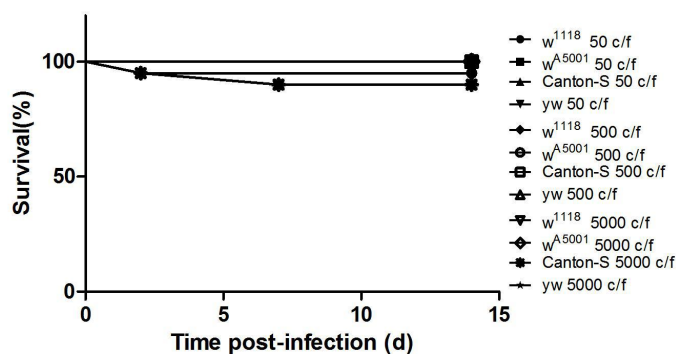
Figure 3



Supplement figures and figure legends

S1

A



B

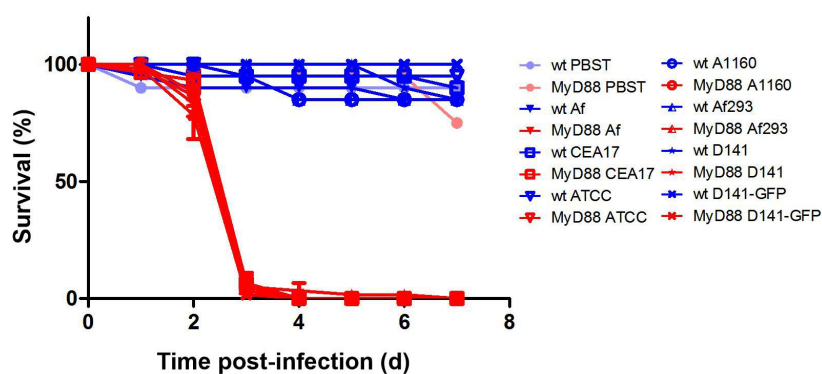


Fig. S1: Wild-type flies dose response and test of different *A. fumigatus* strains in *MyD88* mutant flies.

(A) Survival of indicated wild-type flies exposed to several doses of injected *A. fumigatus* conidia. There was no significant difference in survival between control flies injected with PBST (Phosphate buffer saline containing 0.01% Tween20) and flies injected with indicated doses of *A. fumigatus* ($P = 0.9880$).

(B) survivals of *MyD88* flies exposed to different wild-type *A. fumigatus* strains by injection. There was no significant difference in *MyD88* flies injected with 250 conidia per fly of indicated *A. fumigatus* strains ($P = 0.8753$).

Data were analyzed using log-rank test. All experiments have been performed at least three independent times.

S2

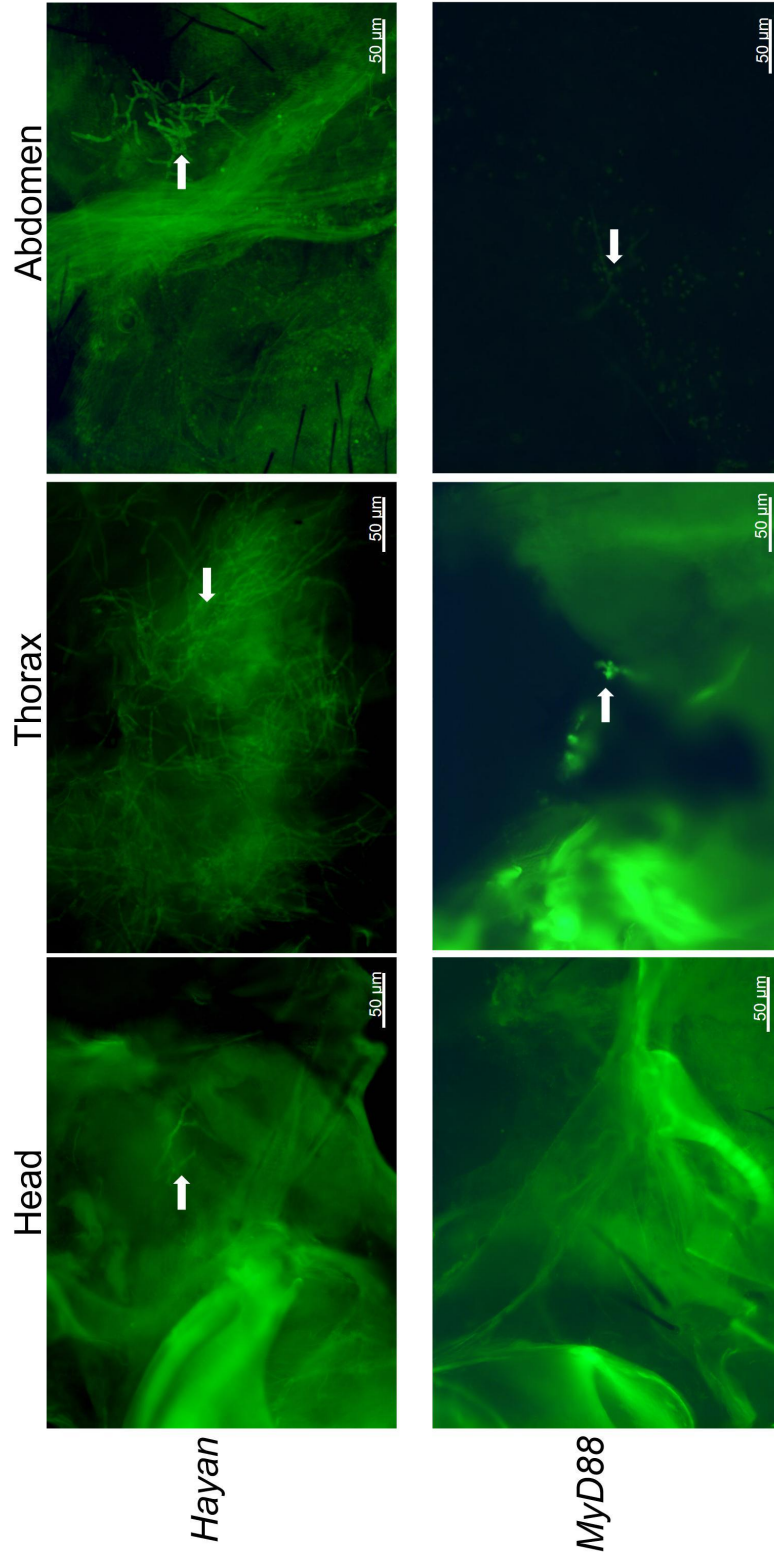
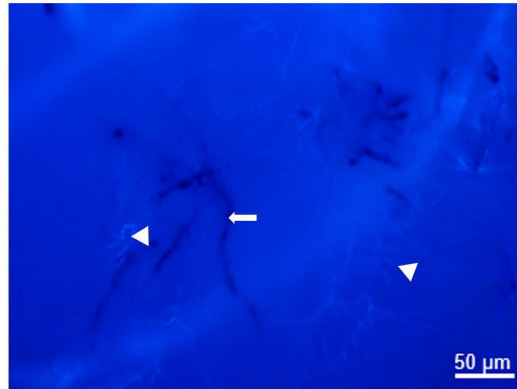


Fig. S2: Fluorescent hyphae in *Hayan* vs. *MyD88*, fluorescent hyphae observed in head, thorax, and abdomen in *Hayan* flies (arrows), whereas observed only in thorax and abdomen in *MyD88* flies (arrows).
All experiments have been performed at least three independent times.

S3

A



B

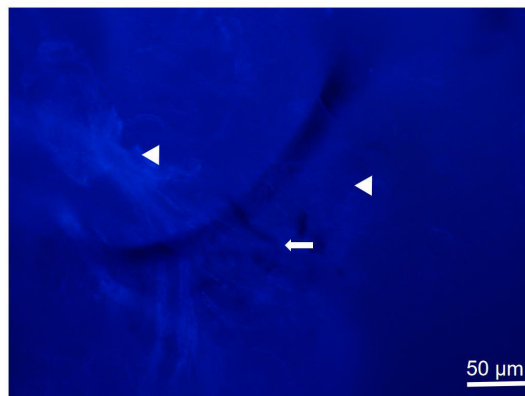


Fig. S3: Hyphae of *ΔpptA A. fumigatus* in *wt* and *MyD88* flies.

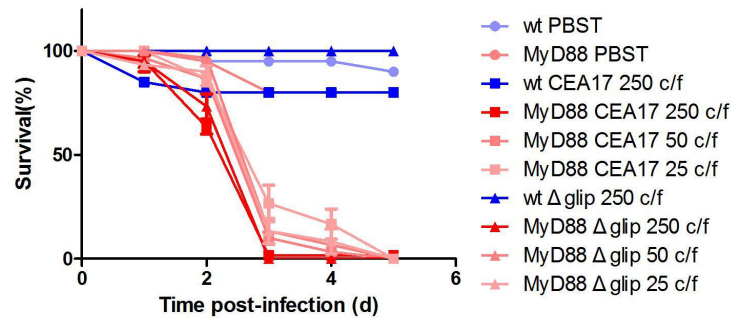
(A) Hyphae of *ΔpptA* (secondary metabolites free mutant) *A. fumigatus* observed in thorax of *wt* flies (arrow) after UVITEX negative staining, sacs and tracheae stained by UVITEX (arrow heads).

(B) Hyphae of *ΔpptA A. fumigatus* observed in thorax of *MyD88* flies (arrow), sacs and tracheae stained by UVITEX (arrow heads).

All experiments have been performed at least three independent times.

S4

A



B

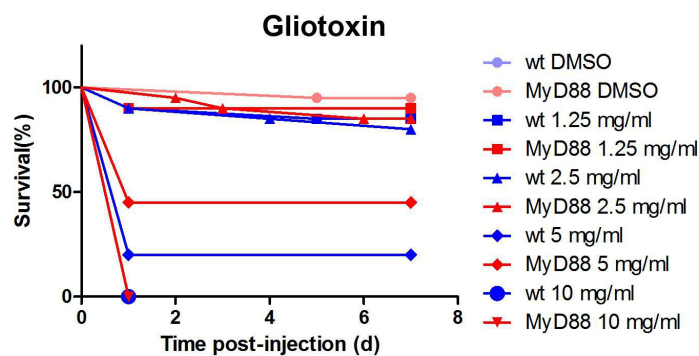


Fig. S4: Gliotoxin is not required nor sufficient to differentially kill *MyD88* mutant flies.

(A) Survival of *MyD88* and wild-type (*wt*) flies exposed to several doses of injected $\Delta gliP$ (gliotoxin free mutant) and (*CEA17* \DeltaakuB^{Ku80} , background of $\Delta gliP$) *A. fumigatus* conidia. There was no significant difference in survival between *MyD88* flies injected with $\Delta gliP$ *A. fumigatus* and those injected *CEA17* *A. fumigatus* with indicated doses of 250 conidia per fly (c/f) ($P = 0.9216$), 50 conidia per fly ($P = 0.9027$), 25 conidia per fly ($P = 0.6883$).

(B) Survival of *MyD88* and wild-type (*wt*) flies exposed to several doses of injected gliotoxin. There was no significant difference between wild-type flies and *MyD88* flies with injected indicated doses 1.25 mg / ml (6.25 ng per fly, $P = 0.6443$), 2.5 mg / ml (12.5 ng per fly, $P = 0.6814$), 5 mg / ml (25 ng per fly, $P = 0.1199$), 10 mg / ml (50 ng per fly, $P = 1$) of gliotoxin.

Data were analyzed using log-rank test. All experiments have been performed at least three independent times.

S5

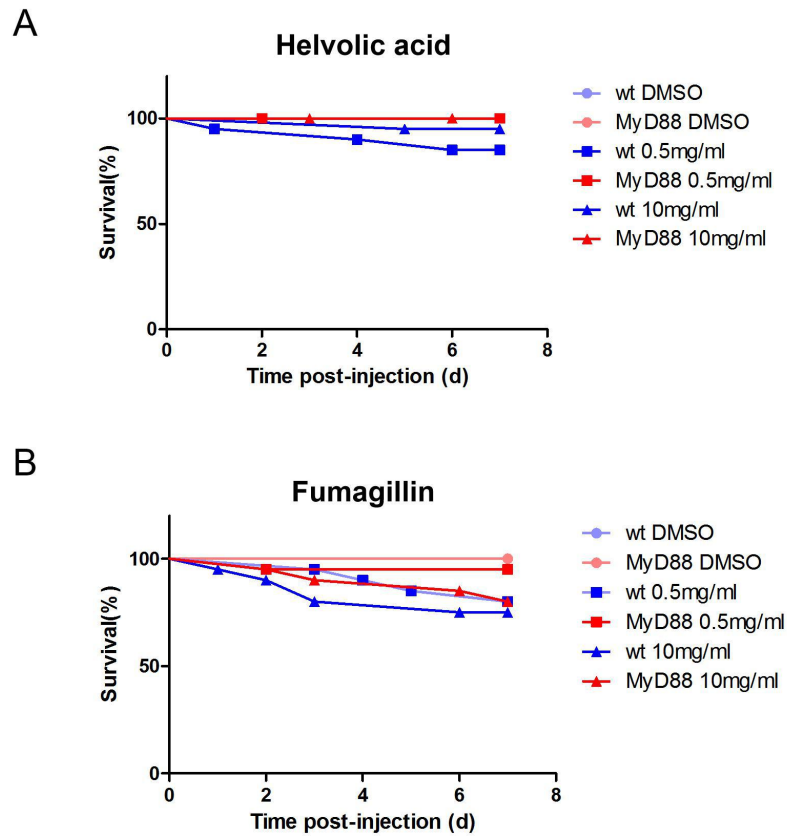


Fig. S5: Helvolic acid and fumagillin are not required for killing flies.

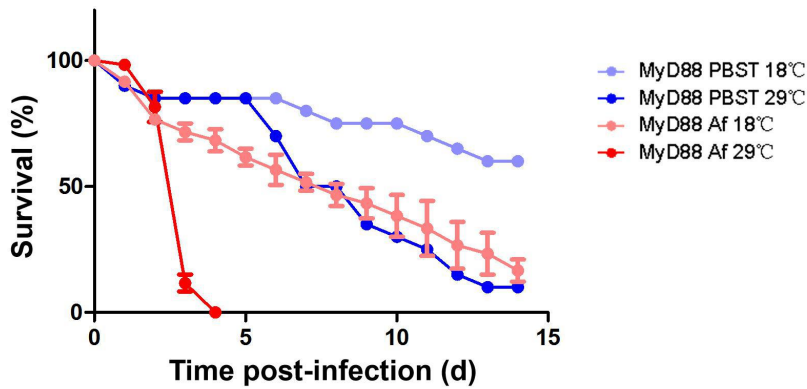
(A) Survival of *MyD88* and wild-type (*wt*) flies exposed to several doses of injected helvolic acid. There was no significant difference between wild-type flies and *MyD88* flies with injected indicated doses 0.5 mg / ml (2.5 ng per fly, $P = 0.0806$) and 10 mg / ml (50 ng per fly, $P = 0.3173$) of helvolic acid.

(B) Survival of *MyD88* and wild-type (*wt*) flies exposed to several doses of injected fumagillin. There was no significant difference between wild-type flies and *MyD88* flies with injected indicated doses 0.5 mg / ml ($P = 0.1689$) and 10 mg / ml ($P = 0.6995$) of fumagillin.

Data were analyzed using log-rank test. All experiments have been performed at least three independent times.

S6

A



B

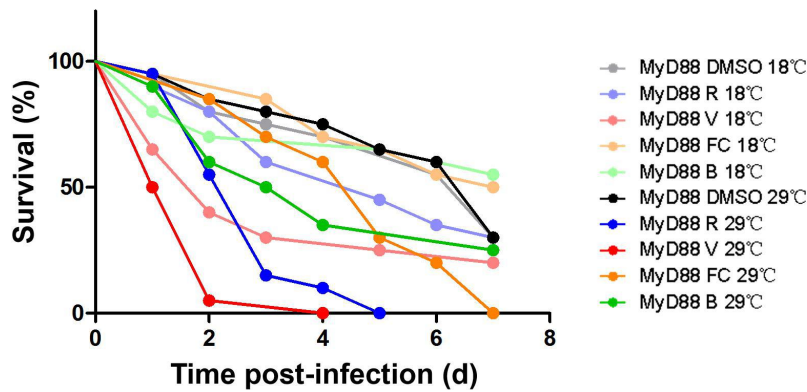


Fig. S6: Temperature is a relevant factor of *A. fumigatus* infection and injection of toxins.

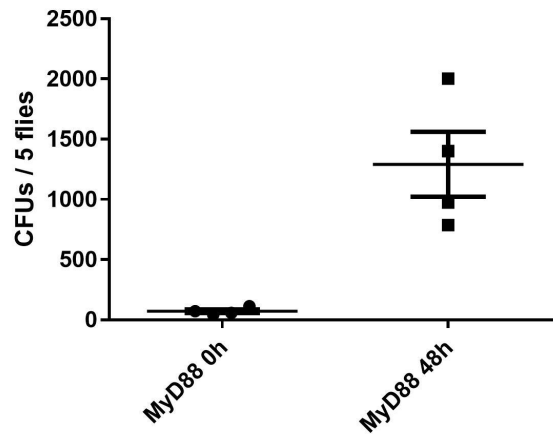
(A) Survival of *MyD88* and wild-type (*wt*) flies exposed to 500 conidia per fly of injected *A. fumigatus* in different temperatures. *MyD88* flies after the injection of *A. fumigatus* and kept in 29°C are died faster than those in 18°C ($P < 0.0001$).

(B) Survival of *MyD88* mutant flies injected with restrictocin (R, 5 ng per fly), verruculogen (V, 5 ng per fly), Bromocriptine mesylate (B, 150 ng per fly), fumitremorgin C (FC, 5 ng per fly) in different temperatures. *MyD88* flies after the injection of restrictocin ($P = 0.0006$), verruculogen ($P = 0.0139$), fumitremorginC ($P = 0.0019$) and kept in 29°C are died faster than those in 18°C, however *MyD88* flies injected with Bromocriptine mesylate ($P = 0.0919$) are similar in both temperatures.

Data were analyzed using log-rank test. All experiments have been performed at least three independent times.

S7

A



B

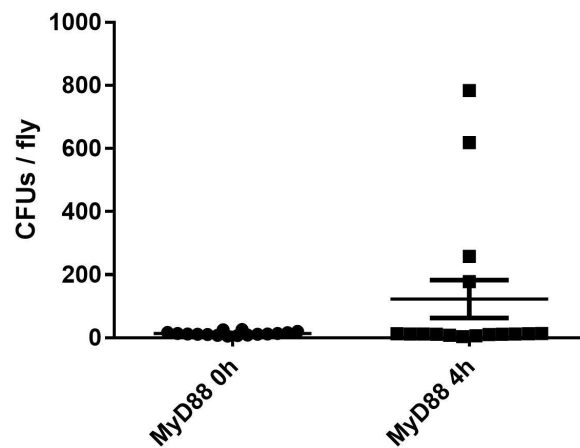


Fig. S7: Bacterial load in hemolymph of fly to check if there is bacterial proliferation during *A. fumigatus* infection.

(A) Bacterial burden was measured at the indicated time points using a plating assay (cfu: colony forming unit) after injection of *A. fumigatus*, 5 *MyD88* flies for each sample. The bacterial load of 48h after injection is higher than 0h's ($P = 0.0286$).

(B) Bacterial burden measured at the indicated time points using a plating assay (cfu: colony forming unit) of single *MyD88* flies after injection of *A. fumigatus*. There are only 25 percent (4/16) samples of the bacterial burden increased in the single fly test, which implied bacterial contamination happened but only minority after injection.

All experiments have been performed at least three independent times.

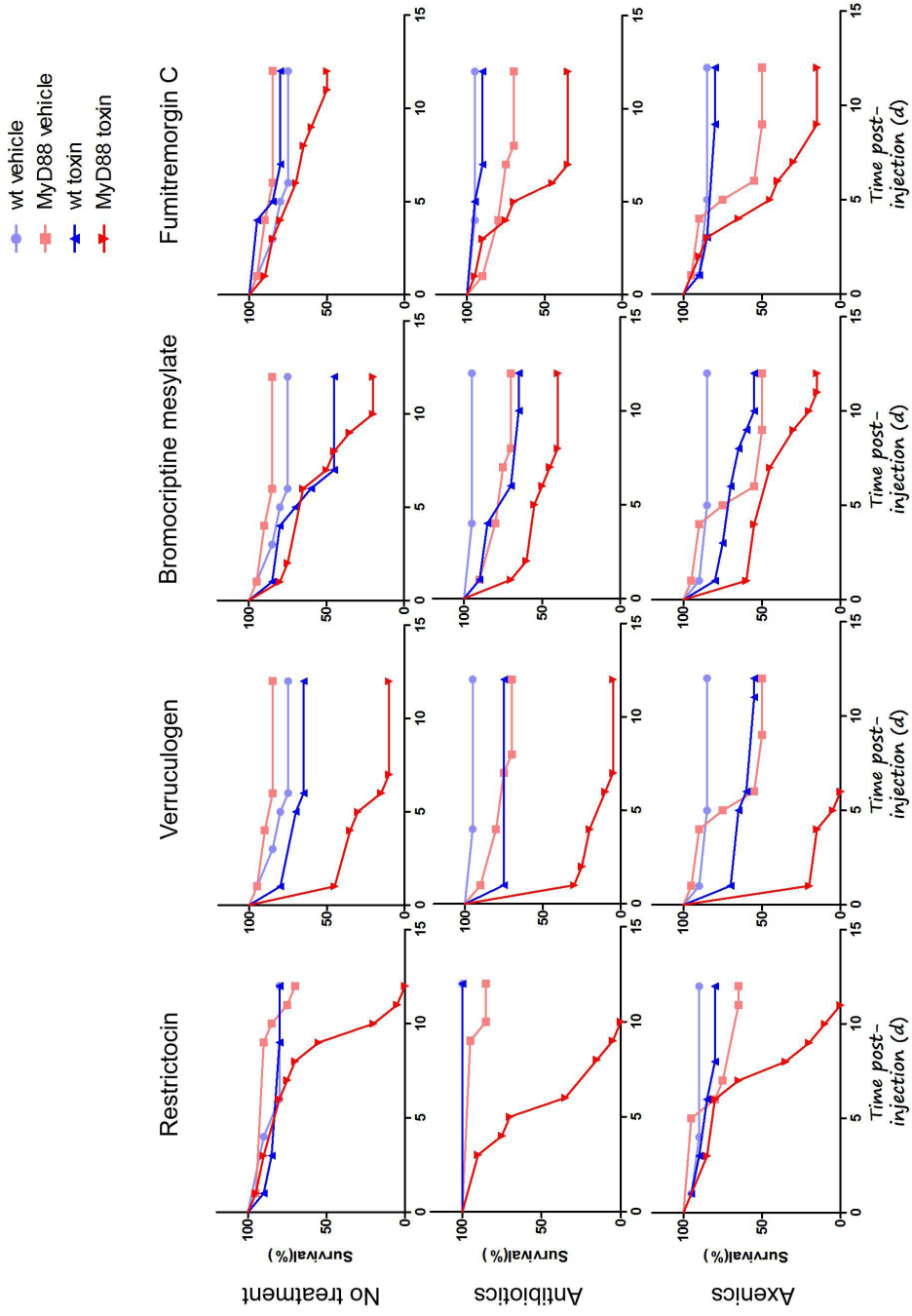


Fig. S8: Antibiotics and axenic flies tests. *MyD88* flies and wild-type (*w^t*) without any treat (No treatment), the antibiotics treated (AB) are the normal *MyD88* mutant flies been treated by antibiotics mixture for two generations and been tested no bacteria left in their body, and axenic flies are the normal *MyD88* mutant flies been treated and proved microbiota free, PBST (Phosphate buffer saline containing 0.01% Tween20) was the vehicle control for restrictocin, DMSO (Dimethyl sulfoxide) was the vehicle control for verruculogen, Bromocriptine mesylate, fumitremorgin C. Survivals of no treatment, AB and axenic *MyD88* flies injected with restrictocin, verruculogen, Bromocriptine mesylate, and fumitremorgin C have similar trend. All the experiments were triplicated at least.

Table

Table 1

	head	thorax	abdomen
<i>MyD88</i>	0% (0/30)	50% (50/100)	1% (1/100)
<i>Hayan</i>	42.5% (17/40)	100% (40/40)	95% (38/40)

Table 1: Quantification of the hyphae exist positions after infection. The table shows the rate of position which found fluorescent hyphae in both *MyD88* and *Hayan* mutant flies, D141-GFP strain was used for injection.

Chapter 2 An unbiased genome-wide RNAi screen on *Aspergillus fumigatus* infections in *Drosophila melanogaster*

Foreword

Chuqin Huang and Rui Xu have collaborated on a large-scale unbiased screen launched at SFHI to identify genes involved in host defense against some fungal and viral infections. This represents a major undertaking of two SFHI teams and is by its very nature a collaborative effort. Rui Xu has developed the *A. fumigatus* infection conditions and optimized various parameters in the pre-screen. He started the large-scale primary screen and performed the first round of retests of the beginning of the screen. Chuqin Huang was trained by Rui Xu. She took the primary screen over and performed a substantial fraction of the retests described in this Chapter. As this is a common project, it would make little sense for each to write a separate Chapter to describe this screen. Rather, data have been pooled and the Chapter has been written together.

Introduction

Large-scale screens are at the heart of the power of *Drosophila* genetics and are critical for a thorough understanding of a biological process by identifying the players involved in it. Ideally, it should be genome-wide. This type of strategy, as it makes no presupposition as to what type of genes might be involved in the process limits some of the biases made in other approaches. When studying host defense to infections, several endpoints can be chosen. For instance, first or second-generation screens were monitoring the induction of the Toll and IMD pathways using reporter transgenes (171, 172). One might also decide to monitor the microbial load. However, not all pathogens are effectively tackled by the systemic immune response, a consideration of special importance in the case of *A. fumigatus*, and the microbial load is technically time-consuming to determine and again, not so relevant in the case of *A. fumigatus*. Third generation screens monitor the survival to infection. The question asked is as follows: is this gene required in host defense against this pathogen? This strategy was not manageable when chemical mutagenesis was used, as it was too difficult to genetically map a mutation using survival assays. One advantage of monitoring survival is that it should allow us identifying genes involved in the host resistance and resilience to infections. Monitoring death as an endpoint is less biased; however, one should always keep in mind that there are many causes that lead to death and that a line exhibiting an enhanced susceptibility may do so because of an indirect effect of the mutation. In some cases, some mutant flies may succumb rapidly to the infection,

as for instance *MyD88* mutants after *A. fumigatus* challenge, even though it does not play a major role in preventing the dissemination of the fungus throughout the body. Conversely, a mutant that affects the dissemination of the fungal pathogen may nevertheless succumb only slowly, as is the case for *Hayan* mutant flies (Chapter 1). It is thus essential to have gained a good understanding of the basic biological processes that underlie infection by a given pathogen. For instance, the first survival screen performed at the genome scale used survival to ingested *Serratia marcescens* as a read-out (71). A significant proportion of hits corresponded to "long-lived" phenotypes in which mutant flies survived longer to the ingestion of these bacteria, and about a quarter of those were related to metabolism. It was understood only later that flies in this infection model succumb to the conjunction of the pathogen and of starvation as the bacteria on the filter compete with flies for sucrose. Also, the researchers were unaware of an important process, which takes place early on only for a few hours, the extrusion of the apical cytoplasm of enterocytes followed by a rapid recovery (85). An important limitation of screens using chemical mutagenesis or transposon insertion lines is as follows. One third of genes are essential and thus there are simply no null mutant adults, as the mutants succumb during development. To bypass this problem, a powerful approach consists in expressing only at the adult stage a transcript that will form an RNA hairpin designed to target specifically a given gene by RNA interference. To this end, we have been using the UAS-Gal4 system, which has been modified so as to allow controlling its activity through the temperature of incubation of the fly line through the use of a thermosensitive Gal4

repressor known as Gal80^{ts}. Several RNAi libraries are now available: two in Vienna based on the dsRNA interference pathway (99), one in Japan, and one in the US based on miRNA interference (TRiP lines), which was developed by Prof. Ni when he was a post-doctoral researcher in the laboratory of Prof. N. Perrimon (173). As obtaining fly lines from abroad in China may be unexpectedly difficult, and as the large-scale screen requires thousands of lines, it was decided to start initially the screen with the collection established by Prof. Ni at Tsinghua University. This collection contains currently some 6400 lines. Limitations of the RNAi line strategy include off-target effects, which are limited in the TRiP library, and a variable efficiency of knock-down of target gene expression. Proteins that are very stable and thus have a low turnover may also be difficult to affect significantly. Finally, this strategy rarely allows uncovering unambiguously the genetic null phenotype. However, processes that involve redundant homologous genes are unlikely to be missed and genome-wide screens have yielded important results.

Implementation of the screen

A large-scale screen mobilizes important human resources, time, and requires an efficient organization to ensure optimized logistics. The ordering of a large number of lines has also a cost. Thus, it makes sense to improve the output of the process by testing multiple pathogens in parallel. Prof. Ferrandon's group is interested in understanding fungal infections while that led by Prof. Peng focuses on viral infections. Thus, two fungal pathogens have been selected: the medically relevant *A. fumigatus*, which is not a major pathogen of *Drosophila* since it cannot kill wild-type flies, and the entomopathogenic fungus *Metarhizium anisopliae*, which is used as a biocontrol agent, of mosquitoes for instance. Importantly, the latter can infect flies by crossing the cuticle once spores are deposited on its surface or can be introduced through septic injury. Here, the first solution was chosen as it may be occurring more often in nature, and that we will refer later to as “natural” infection. Three viruses have been selected for testing in the screen, *Drosophila C virus*, a natural fly pathogen, Sindbis virus, which can infect both invertebrates and vertebrates, and Dengue virus, a medically-important arbovirus.

The genetic scheme used in the screen is shown in **Fig. 1**. The principle is to cross several virgin females collected in a driver line stock that has been amplified to males sorted from the library collection. The cross is made at 25°C for three days to ensure efficient fertilization of the virgin females by males. The adults are then transferred to another tube while the tube containing the eggs is moved to 18°C to bypass developmental lethality by shutting off the expression of the driver line (the Gal80ts

repressor is active), although some embryos that have begun their development at 25°C may already have been killed when essential genes are targeted, hence lowering the amount of progeny in this case. Once development is achieved and that the adults have hatched, the vials are moved to 29°C for five days prior immune challenge to initiate the RNA interference (RNAi). At this step, flies are sorted and the males discarded, as the assays are more sensitive when performed on females (*M. anisopliae*), which are easier to inject and more resistant to injury. In the case of *A. fumigatus*, the fungus is sensitive to one of the preservatives added in the regular food, in this case sorbitol. For this reason, since the flies were kept on regular fly food before infection, they were transferred to tubes containing only a sucrose solution for two days so that the sorbitol was washed out of the fly organism. After the infection, the flies were transferred on fly food without sorbitol for the duration of the survival experiment.

Even though the crossing scheme is in principle very simple as it involves a single cross, it nevertheless requires an optimization of each single parameter that then need to be put together and tested under real conditions. This is the reason why a pre-screen step was first implemented. It helps training the investigators and identifying problems that may not have been foreseen. One of its goals is also to establish the maximal capacity of the organization in terms of the number of lines that needs to be processed. The aim is also to establish an invariable schedule that will remain the same throughout the duration of the screen.

An important step is thus to establish a list of all of the parameters that are involved in the screen, from ordering of the mutant stocks to the management of stocks once they have been tested, including space and incubator issues (see below the section about the technical aspects of the screen).

Results from the screen

After the pre-screen, we refined the genetic scheme with more details (**Fig. 2**), and then started the primary screen. A survival curve of a batch of flies with relatively few lines assayed in parallel from the primary screen is shown as an example on **Fig. 3**. We also did some retests during the primary screen to check the efficiency. In 25 months, we screened 6741 lines in the primary test, and performed 1154 retests (**Table 1, 2**). 245 interesting hits were sorted from retests (about 20% confirmation rate from primary test to retest), the degree of severity of their phenotype having been scored from one to three stars. The genes scored at least two stars are considered to give a strong phenotype. There are 57 genes with 2 stars (**Table 3**), 5 genes with 2.5 stars (**Table 4**), 9 genes with 3 stars (**Table 5**), and the remaining hits gave a weak phenotype (**Table 6**). There are 151 lines that have a shortened life span and are likely essential for viability, 87 lines sensitive to wound, and 671 lines for which the phenotype was not confirmed (see **Fig. 3** for examples of phenotypic categories observed during the screen). Of note, we found *CrebA* as a strong hit in our screen. Interestingly, a study of fly transcriptomic response to 10 bacteria was published right after our discovery, in which *CrebA* was described as an important gene for resilience to infection (174). This result partially validates our screen (**Fig. 4**). Rui Xu has further validated the CycK hits and associated genes (Chapter 3) and Chuqin Huang has performed a thorough work on the Mediator complex, several subunits of which were picked-up in the screen (Personal communication).

We made an overlap analysis to the hits obtained in the parallel screens with the different microbes in this project, except for dengue virus which did not give any hit in the retest so far (**Fig. 5**). To our surprise, the two fungi did not overlap much with 27 common hits, but around 1/5 of *A. fumigatus* hits, that is 36 genes, are overlapping with *Drosophila C Virus* (DCV) hits. This implies that the host response to fungal infection is very different between *A. fumigatus* and *M. anisopliae*. According to our experience in fungal counts after infection, we found that *A. fumigatus* is not proliferating much in the *Drosophila* body, in contrast to *M. anisopliae* that proliferates intensely in the final 24 hours of the infection. *A. fumigatus* virulence is likely to depend more on its secreted secondary metabolites and their mycotoxins than on colonization, perhaps explaining the difference we observed. In addition, the RNAi screen was done by injection for *A. fumigatus*, although *M. anisopliae* infection was performed by natural infection. This may also lead to a different behavior from the host response.

Gene ontology (GO) analysis of *A. fumigatus* hits shows that the regulation of transcription by RNA polymerase II is the strongest related process, followed by actin filament-based process (**Fig. 6A**). Of note, the enrichment of genes involved in regulation of transcription by RNA polymerase II does not simply reflect the identification of Mediator complex subunits, since only two of them were found as hits through this screen. We got some groups that were expected, like regulation of response to stress, but few genes involved in classical immunity (*bona fide* immunity hits were *Grass* and *Dif*). The actin filament-based process, membrane trafficking and

endocytosis may be related to cellular immunity. Regulation of transcription and transport of small molecules would allow the secretion of soluble antifungal effectors. The fact we did not find many immunity-related genes can be due to the redundancy of the effectors, meaning that the knockdown of one of them would not affect enough the global immune response of the host. Another possibility is that we may have identified some genes involved in new resilience mechanisms, that would make them interesting to further study.

In the few common genes between the two fungi, we can find *Grass*, a serine protease involved downstream of *GGBP3* and *PGRP-SA* in the activation of the Toll pathway, that was expected as *Grass* is playing a role downstream of fungal recognition factors (**Fig. 5B**). Two genes, *Splenito* and *CG34404*, are involved in the wingless (*wnt*) pathway: this signaling pathway may be part of a yet uncharacterized antifungal mechanism that could be further analyzed in the context of fungal infection. An inhibitor of the Jak-Stat pathway, *Su(var)2-10*, is also involved in both fungal infections (**Fig. 5B**).

Finally, the GO analysis also showed there is some difference between the hits giving strong and weak phenotypes. Hits with a strong phenotype are more related to regulation of transcription by RNA polymerase II, multicellular organismal process, locomotion, and actin filament organization (**Fig. 6B**), but membrane trafficking, stem cell population maintenance, protein phosphorylation related genes are more important for the hits with a weak phenotype (**Fig. 6C**). These weak signals may be worth pursuing. For instance, there are only two active types of stem cells in the adult

female fly, stem cells in the gonads and intestinal stem cells. It may be worth determining whether *A. fumigatus* infection impacts oogenesis and whether it may affect the homeostasis of the intestinal epithelium by measuring the proliferation rate of intestinal stem cells.

Conclusions

The results presented in this Chapter should still be considered as tentative as more confirmations are needed to validate the hits. The degree of validation shall depend on the importance of the mutant as determined from the severity and reproducibility of the phenotype as well as the biological process in which the hit is likely to be involved. For instance, hits affecting the nervous system may be especially interesting in that they may mediate the resilience to fungal mycotoxins that target the host nervous system. Independent confirmation using other mutants, including CRISPR-Cas9 null mutants if the gene is not essential, and if possible genetic rescue by a wild-type copy of the gene carried on a transgene will be central to the final validation process, using specific-pathogen free lines to exclude a susceptibility to an opportunistic infection. A thorough phenotypic analysis will be pursued on the most interesting hits, including determining the susceptibility to other pathogens, determining whether the Toll pathway is impacted or not, whether the mutants are sensitive to injected mycotoxins, investigating the fungal load, determining the tissue in which the hit is important using tissue-specific drivers, *e.g.*, using neuronal, hemocytic, or fat body-specific drivers of the UAS-RNAi transgene.

With these limitations in mind, one striking result is that flies with an apparent intact immune system as we understand it today are sensitive to *A. fumigatus* infection. They may affect resilience to infection rather than resistance. When placed in the framework of our current understanding of *A. fumigatus* infection in *Drosophila*, that

is that resilience to toxins is a critical host defense, it will be important to determine whether these mutants are able to withstand the injection of mycotoxins.

At present, the screen has essentially tested the Tsinghua Trip collection from Prof. Ni. As more lines have also been generated in Japan, and as all lines are centralized in Boston and then made available through the Bloomington stock center, the current effort is to obtain these lines so as to exhaustively test this resource. We shall however be far from saturation, that is from having tested each gene of the genome. Furthermore, some important host defense processes may be difficult to identify through RNAi analysis, for instance if it involves stable proteins. Thus, it will always remain a possibility that we have missed an essential host defense system. Nevertheless, the more lines will have been screened, the more valuable this resource will be to understand host defense against fungal infections.

In the long term, the most interesting hits will need to be tested in a murine infection model of invasive aspergillosis if the corresponding gene has been conserved during evolution. Even though the adaptive immune system is able to generate antibodies that may neutralize the action of antitoxins, it is however a process that may be too slow to efficiently fight invasive aspergillosis, especially in neutropenic patients. Thus, it is a stimulating prospect that an innate resilience mechanism against mycotoxins may have been conserved during evolution and that our large-scale genetic approach may help uncover it.

Technical aspects of the screen

In the following, we describe in detail how the screen was organized and give the parameters that have been chosen to implement the screening strategy following the pre-screen. As the logistics of the screen are important given the number of lines analyzed and the five infection screens performed in parallel, fly handling (stock ordering and maintenance, collection of virgin females, genetic crosses) was essentially performed by a highly competent team of technicians and the actual infections, as well as data analysis and decisions about whether a given line should be retested or considered to be negative in the primary test, were performed by students distributed in distinct teams according to the tested pathogen.

Technical details and Organization:

1. Three males of UAS-RNAi flies with 6 Ubiquitin-Gal4,tub-Gal80^{ts} virgins per tube were crossed at 25 °C, this on 4 tubes in parallel. After 3 days, the adults were transferred into new tubes kept at 25 °C, whereas the tubes containing the offspring of the cross (eggs and first to second instar larvae) were placed at 18 °C. The adults were transferred an additional time so as to obtain in total three batches, the first one being used for actual test and the two other ones as back-ups kept at 18 °C. Each batch of the primary test included usually about 600 random lines. Since 4 tubes were needed for each line, each tube with 6 virgins, 14,400 Ubiquitin-Gal4,tub-Gal80^{ts} virgins were needed per batch each week, a massive undertaking.

2. After 3 weeks at 18°C, most of the pupae hatched. 25 females were sorted in five vials, one per pathogen to be tested in the primary screen, which did not include any controls to make it manageable. 125 females were required for retests of a given pathogen altered survival phenotype since this time three survival to infection were performed in parallel; also, one uninfected control was added to determine if the gene is essential for survival, and another one additional control in which flies were injected with buffer to assess whether the gene might be required to withstand the effects of a wound. Each batch of primary test involves 600 lines, so $25 \times 5 \times 600 = 75,000$ flies were needed to be sorted for each batch. Thus, more than 750,000 flies have been sorted for the primary test up to now. The number of retested lines depends on the results of primary test, so it will be tens to hundreds of lines for each pathogen.

3. The next step consisted in placing the sorted RNAi mutant flies at 29°C for 5 days to express the RNAi transgene and to leave it the time to act on its target genes, at least those with a significant protein turnover during this period. In the case of *A. fumigatus*, the flies were kept for three days on fly food, and then for two days on 100 mM sucrose solution deposited on filter paper. These specific tubes were prepared by the members of the *A. fumigatus* group.

4. The *A. fumigatus* suspension needs to be prepared fresh, at most 6h prior to injection; therefore, at least 10 plates of *A. fumigatus* were always prepared, 5 for direct use, and the others as backup. Furthermore, frozen conidia were kept at -80°C as the backup. The advantage of this strategy is that the conidia were in an excellent physiological state. However, a drawback of this approach is that the virulence of teh

conidia may vary according to the batch. When signs of degeneration or contamination of our *A. fumigatus* stock were detected, the cultures were started afresh from novel frozen aliquots of the fungus.

5. 600 lines were injected in one week, the infected flies were then placed again on fly food, and then their survival was recorded each day for the following 2 weeks. During the survival recording, fly food needed to be changed every 3 days. Each batch of primary screen needed $600 \times 5 = 3,000$ small vials of food. UAS-mCherry crossed with Ubiquitin-Gal4, tub-Gal80^{ts} virgins constituted the wild-type (negative) control for all pathogens and were prepared by the technicians. In contrast, *MyD88* flies (the positive control) were prepared by the fungal screening team.

6. The survival data were entered in a computer and analyzed after drawing of survival curves. According to the results of pre-screen, *A. fumigatus* cannot kill wild-type flies, so it was relatively easy to judge if the lines were interesting or not by monitoring their death rates after two weeks. Actually, more than 80% of the tested UAS-RNAi lines displayed a survival rate inferior to 80%. Sometimes, the wild-type control flies also succumbed at a 10-30% rate, reflecting a long observation period of two weeks after injection. In the pre-screen, around 10% lines got a survival lower than 50%. In addition, 50% is a classical threshold for survival, so we set 50% as the threshold for deciding whether a line should be retested.

Note: Step 1, 2 were performed by the technician team whereas the other steps were performed by members of the different pathogen testing groups.

Logistics:

1. UAS-RNAi lines ordering. Prof. Ni's laboratory prepared 200 lines per week randomly from their collection and shipped the lines to SFHI. Technicians received and took care of them, checked their condition and recorded the lost lines to be reordered. When some shipments got lost during shipment, some technicians then went to Beijing to transfer the stocks with them.

2. Maintenance of transgenic fly stocks. After the UAS-RNAi lines passed quarantine, the technicians kept them at 18 °C and flipped the stocks each month. There raised a large stock of Ubiquitin-Gal4,tub-Gal80^{ts} flies in parallel, one at 25 °C and another other one in 18 °C as backup. Each stock consisted of around 500 big vials, the quantity necessary to harvest the required numbers of virgins each period of three weeks. Each month, some stocks were checked for the absence of known pathogens: hundreds of samples were collected randomly from both UAS-RNAi lines and Ubiquitin-Gal4,tub-Gal80^{ts} stock, then checked for the absence of Drosophila C virus, Nora virus, microsporidia by RT-qPCR, and Wolbachia by classical PCR coupled to agarose gel electrophoresis. The Trip line stocks were usually pathogen-free, except for the Nora intestinal virus. The Ubiquitin-Gal4,tub-Gal80^{ts} line remained pathogen-free over the course of the screen.

3. Supply of fly food. Each week, the whole screen project necessitated at least 15,000 small vials and 1000 big vials to be prepared, that is, more than 100 l of liquid fly food needed to be prepared. One worker made the food, some technicians and students helped stoppering the vials. All food ingredients were ordered from the same

supplier to keep food quality as consistent as possible during the screen.

4. Dissecting microscopes were used both for fly sorting and for the injection of the pathogens; four dissecting microscopes were dedicated to sorting, and there was at least one dissecting scope for each pathogen. Of note, fungal and viral infections were performed in separate, dedicated rooms. Four dissecting microscopes were present as a backup. There were two Nanoject II Auto-Nanoliter Injectors to perform the injection for each of fungal or viral pathogen.

5. To raise the flies and to perform infections, incubators at three temperatures (18, 25, 29 °C) were needed. Five 400L incubators were used to prepare raise the fly stocks, two of them at 18 °C, one at 25 °C, one at 29 °C, and one as a backup. These incubators were located in the storage rooms. Two incubators at 29 °C were required to incubate the infected flies, one for each infection room (fungus and virus). Technicians prepared the flies in a single room, and stored the Ubiquitin-Gal4,tub-Gal80^{ts} and UAS-RNAi flies in a dedicated 18 °C room.

Quality control

1. Primary screen with either fungi or viruses: survival curves of the bulk as well as that of the positive (immunodeficient flies) or negative (wild-type flies) controls. Of note, as in many immunity large-scale screens, these controls were sometimes erratic; in this case, the mean survival of the tested lines provided an independent parameter: as the lines were random, the expectation is that the average of many lines will represent a measure of the wild-type situation. This also allowed to mitigate the

effects of varying pathogen virulence from batch to batch. In the case of retests, this measure could not be used as the lines were no longer random as they were selected in the primary screen. Thus, for many batches, we used two distinct wild-type lines, one with a UAS-mCherry-RNAi transgene, which we often found to be somewhat sensitive to the infection, and the other our *w^{A5001}* reference stock.

2. Analysis of survival curves: the criteria set for selection a line as a retest depended on the pathogen (*Af*: % of surviving flies at day 14 [wt flies should not be killed]; *Ma*: LT50 as compared to average LT50 of the batch)

3. Retest line with 3x20 flies in parallel, including noninfected controls and mock-injected/infected (PBS for *A. fumigatus*, mock infection with just water: vortexing, going through drying with a vacuum for *M. anisopliae* “natural” infection).

4. Definition of criteria for analysis of retests: comparison to wild-type negative controls (mCherry, A5001: multiple samples), susceptible positive control (multiple samples, at least 5). Most important, the reproducibility with primary screen data was deemed essential, even if the phenotype was weak.

5. Perform a second round of retests, analyzed as above: the important parameter is reproducibility.

6. Procedure to validate confirmed lines after several retest rounds: i) sequence the insert to check that the targeted gene is indeed that reported on the line name and that there was no swapping of stocks; to check the vector used for transformation PCR using appropriate primer set as described below will be performed. The sequence of half of the hairpin will be Blasted to check the location of the target and the sequence

will also be compared to sequences provided either by Prof. Ni (Excel file) or available on the TRiP site (<http://fgr.hms.harvard.edu/fly-in-vivo-rnai>). Finally, off-target predictions on each transgenic line of a selected candidate will be checked.

For VALIUM1, the primers are:

F: 5'-CGCAGCTGAACAAGCTAAAC-3'

R: 5'-CGACTGCGAATAGAACTCAC-3'

For VALIUM10, the primers are:

F: 5'-CGCAGCTGAACAAGCTAAAC-3'

R: 5'-CTAGACTGGTACCCTCGAATC-3'

For VALIUM20 and VALIUM21 the primers are:

F: 5'-CGCAGCTGAACAAGCTAAAC-3'

R: 5'-TAATCGTGTGTGATGCCTACC-3'

For VALIUM22 the primers are:

F: 5'-GGTGATAGAGCCTGAACCAG-3'

R: 5'-AATCGTGTGTGATGCCTACC-3'

7. For weak phenotypes, if an important off-target effect is predicted, another independent RNAi transgene will be tested (optional step: only if not too many); the weak phenotypes are not a priority for analysis, unless many of them are found belonging to a same pathway/biological process. This list is however useful for Gene Ontology analysis to pick up weak signals. Also, we shall look whether the genes are evolutionarily conserved. In the long term, it will be worth determining whether human patients have SNPs close to their homologues (GWAS analysis).

8. For the strong phenotypes, confirm the phenotype by using other independent RNAi lines or existing mutants. Prioritization will take into account on a number of considerations: besides the severity and reproducibility of a phenotype, the intrinsic interest of the gene/biological process needs to be assessed. A first step is to look the gene data on Flybase (when dealing with "metabolic" genes, it is not always easy to determine the accurate biological function; by looking for human homologues in human databases through the links provided by Flybase: HUGO or G2F, then use GeneCards, GeneAtlas. One will also have to take into consideration other available data *e.g.*, in-house RNAseq data for *M. anisopliae* and *E. faecalis*; viruses: Prof. Jean-Luc Imler has performed numerous transcriptomics analyses for many viruses). Also, one will need to check the data from other laboratories, for instance by checking the Reference section of Flybase (this section often indicates Supplemental data/tables of articles). The phenotype in the other parallel screens will also be looked at to gain more insight into the gene's function. If the gene is not obviously lethal, CRISPR-Cas9 mutants from our facility will be ordered to check the phenotype. Besides assays fitted to the nature of the gene, it will be a good policy to systematically investigate the survival to other pathogens not included in the screen, such as Gram-negative bacteria. It will also be important to determine whether signaling pathways are affected and check whether the microbial load is altered, an indication as to whether the gene is involved in resistance or resilience. For genes studied in depth, overexpression and rescue will be desirable as well as determining which tissue/cell type is critical for the gene's role in host defense.

9. As the outstanding result of the analysis of *A. fumigatus* pathogenesis is the importance of the resilience to mycotoxins, it will likely be worth to perform a mini-screen on the strong phenotype mutants to determine which ones are sensitive to the injection of a couple of mycotoxins, restrictocin as it is not a secondary metabolite and cleaves a ubiquitous target, rRNA, and fumitremorginC as it targets the nervous system. It will be a strategical priority to investigate hits from this secondary screen.

Figure legends

Figure 1: Schematic overview of the screen strategy

Figure 2: Scheme of the organization and logistics of the screen

The actions boxed in white were performed by the team of technicians in charge of handling the logistics of the fly stocks and of making the crosses whereas the infection parts were handled respectively by the fungal and viral teams of investigators.

Figure 3: Example of one batch of primary screen. Af 250 conidia/fly injection, blue curves are negative controls, red curves are positive controls, green curves are candidates for retests, black curves are lines without phenotype.

Figure 4: Examples of the different categories of phenotypes encountered during the screen

A. Example of the phenotype of sensitivity to wound: the control flies injected with the PBST buffer die about at the same rate as infected flies; Af 250 conidia/fly injection.

B. Example of the lethal phenotype: the uninfected and buffer-injected controls succumb at the same rate as the infected flies, thus precluding an analysis of the function of the gene in host defense; Af 250 conidia/fly injection.

C. Example of a weak phenotype line: only 50% of the flies succumb after two weeks post-challenge; as this phenotype is weak but reproducible, it was awarded a grade of one star. Af 250 conidia/fly injection.

D. Example of a strong phenotype line: the flies succumbed even faster than the positive *MyD88* control; as the buffer-injected control also succumbed to the injection, but at a much slower rate, this line has been graded only as a two star. Af 250 conidia/fly injection.

E. Example of a line exhibiting no phenotype; Af 250 conidia/fly injection.

Figure 5: Venn diagram of different pathogens overlap analysis.

A. The overlapping hits between *A. fumigatus* (*Af*), *M. anisopliae* (*Ma*), Drosophila C virus (DCV), Vesicular Stomatitis Virus (VSV).

B, C. The overlapping genes between *Af*, *Ma*, DCV and VSV are listed in the tables.

Figure 6: Gene ontology analysis

The Metascape online tool had been used to perform Gene ontology analysis (175)

A. Gene ontology analysis of all 245 interesting hits pooled together regardless of the strength of their phenotypes.

B. Gene ontology analysis of 68 strong phenotype hits ranked with a grade of at least two stars.

C. Gene ontology analysis of 177 weak phenotype lines graded less than two stars.

Table 2: Schedule of the screen.

Table 3: Summary of the results of the *A. fumigatus* screen.

The confirmation rate of lines kept for retest was 21%; about 18% of the lines analyzed during the primary screen were kept for retests. This apparent low degree of stringency in the primary screen allows nevertheless identifying genes with a weak survival phenotype, and occasionally some with a stronger phenotype due to the variations in survival from experiment to experiment. The false negative rate is somewhat difficult to estimate. *One of us, Chuqin Huang, has focused on the genes encoding the Mediator complex subunits for a thorough genetic analysis. By thorough testing in multiple survival experiments, we have identified 19 genes with a reproducible sensitivity to A. fumigatus infection that were missed in the primary screen thus yielding a rate of 16% (# Med subunit genes missed in the primary screen/# of subunit genes screen x 100)*

Table 3: List of hits with rank of two stars

Table 4: List of hits with rank of two and a half stars

Table 5: List of hits with rank of three stars

Table 6: List of weak hits with rank lower than two stars

Figure 1

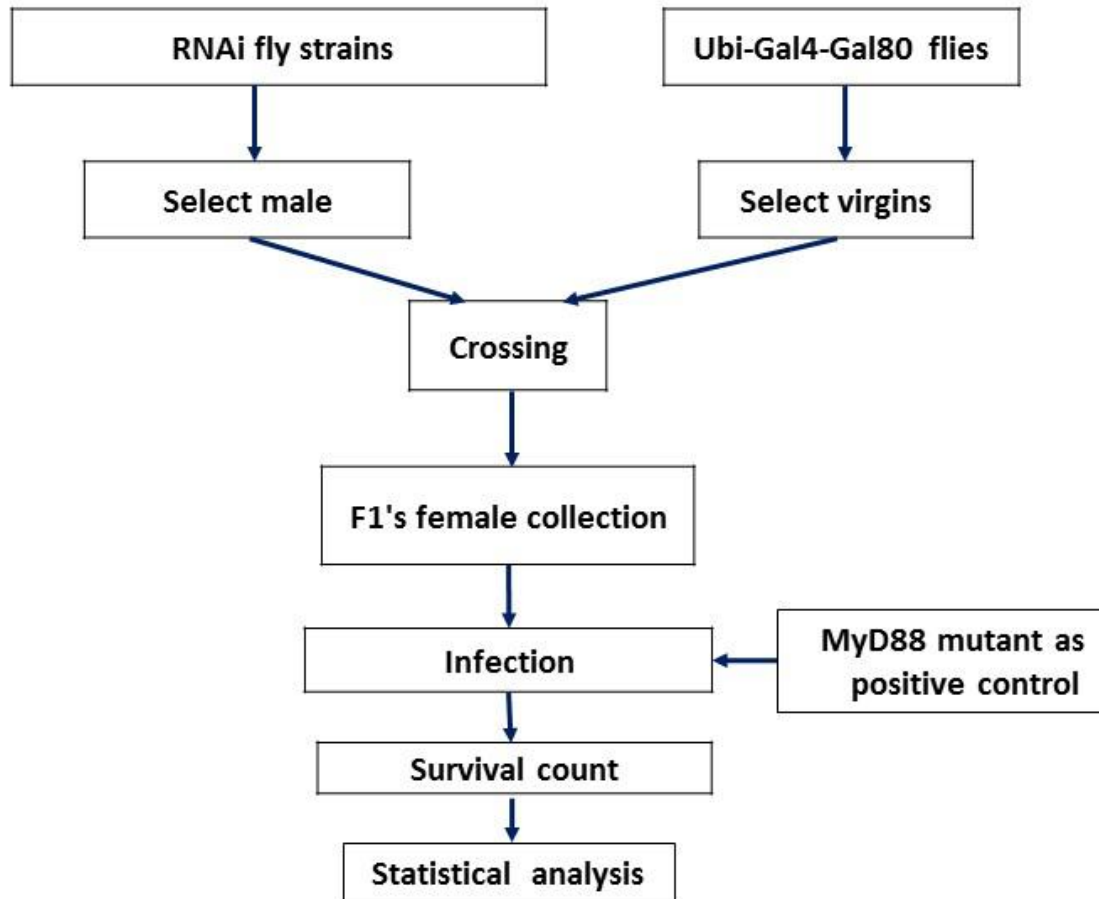


Figure 2

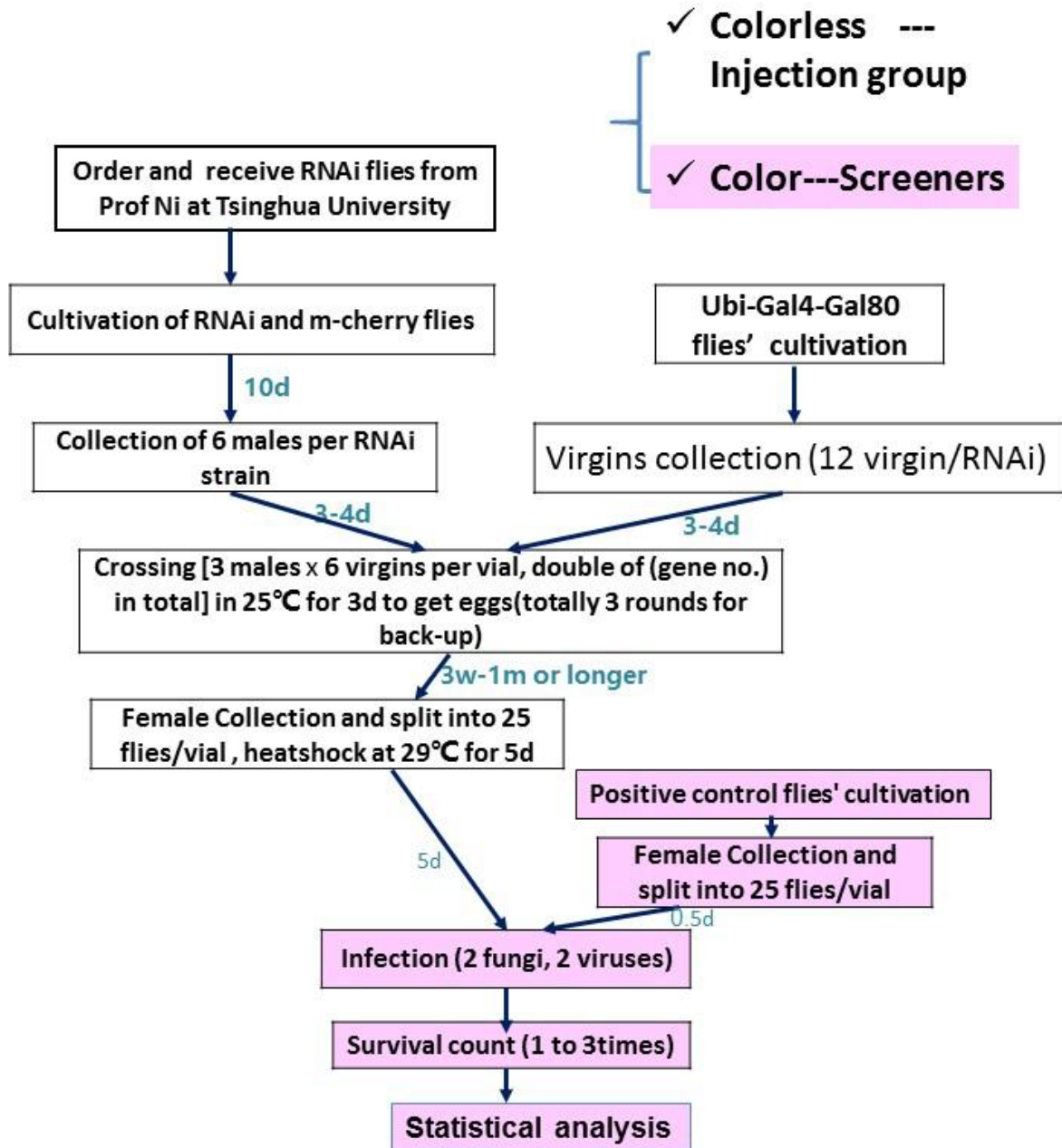


Figure 3

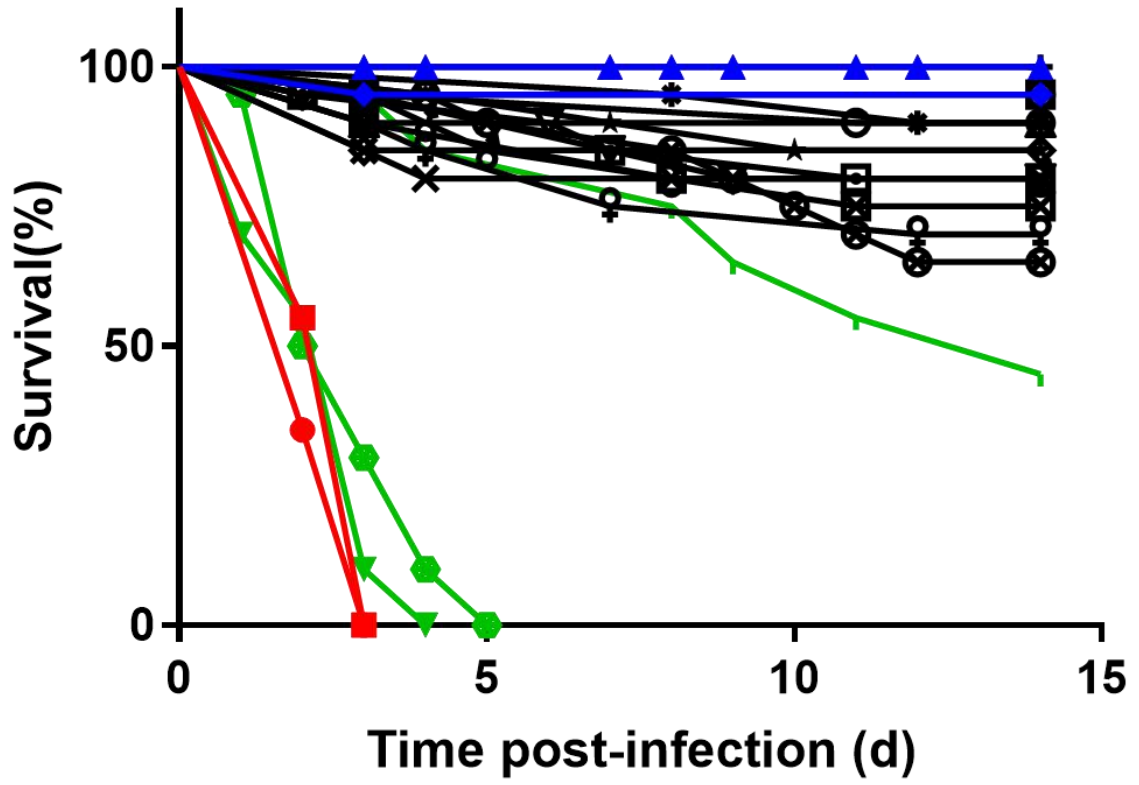


Figure 4

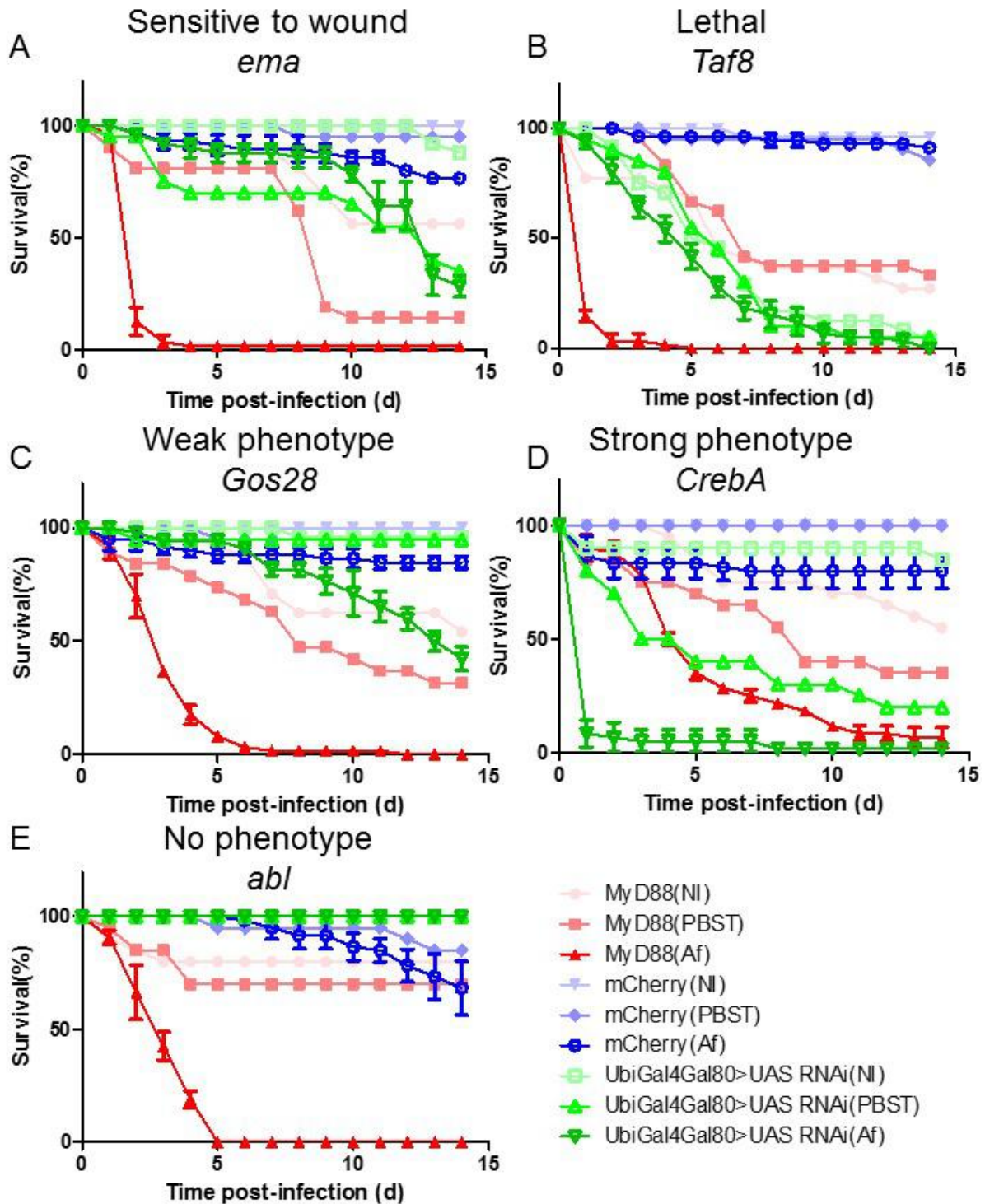
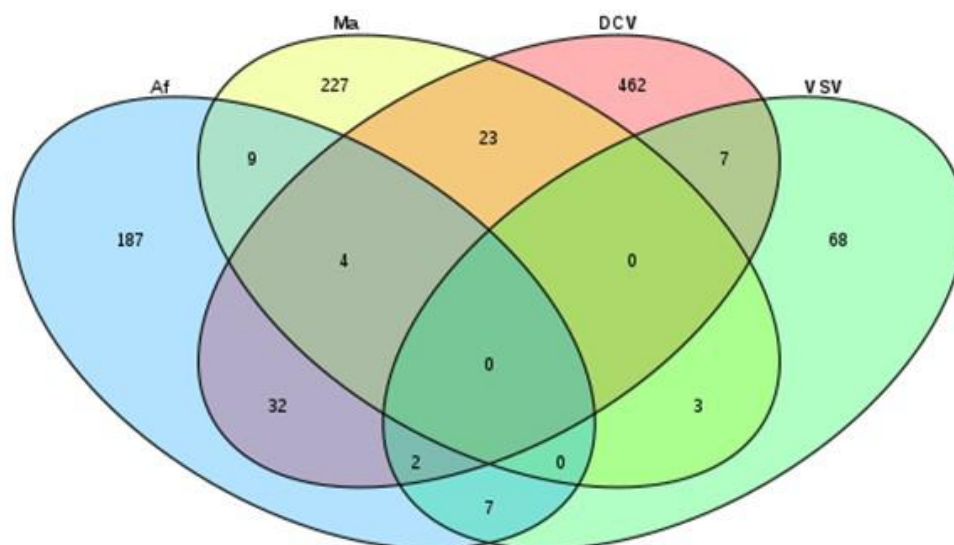


Figure 5

A



B

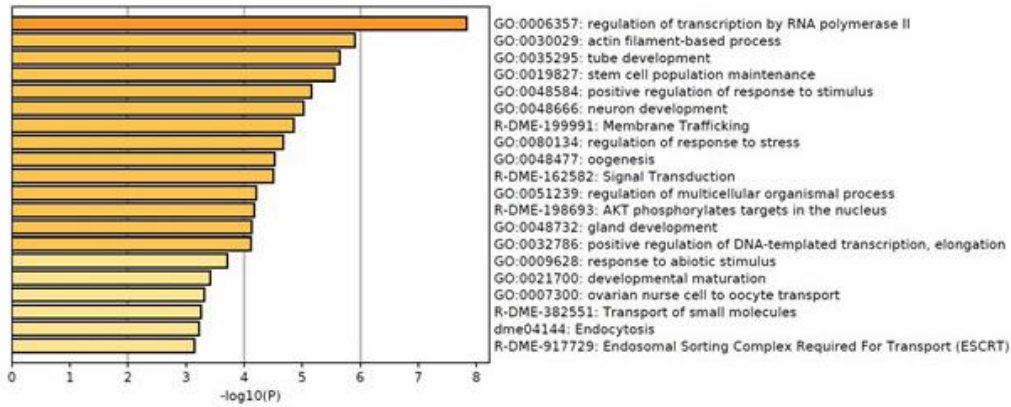
Gene name	Common hits	Gene function
<i>CG6749</i>	<i>Af</i> , DCV, VSV	Human ortholog = IGFALS (insulin like growth factor binding protein acid labile subunit)
<i>RabX5</i>	<i>Af</i> , DCV, VSV	Rab GTPase
<i>Grass</i>	<i>Af</i> , Ma, DCV	Secreted serine protease involved downstream of GGBP3 and PGRP-SA in the activation of the Toll pathway
<i>bb in a boxcar (bbc)</i>	<i>Af</i> , Ma, DCV	Phosphotransferase ; human ortholog = CEPT1; phospholipid metabolism
<i>CycK</i>	<i>Af</i> , Ma, DCV	Cyclin-homologous subunit that forms a complex with the transcriptional kinase encoded by Cdk12
<i>CAH16</i>	<i>Af</i> , Ma, DCV	Carbonic anhydrase
<i>Splento (ntto)</i>	<i>Af</i> , Ma	Methyltransferase; positive Regulator of Wnt-TCF signaling pathway
<i>neuralized</i>	<i>Af</i> , Ma	E3 ubiquitin ligase of the RING family; part of the Notch signaling pathway + Notch-independent functions in epithelial morphogenesis
<i>CG34404</i>	<i>Af</i> , Ma	Human ortholog = MCC regulator of WNT signaling pathway
<i>Su(var)2-10</i>	<i>Af</i> , Ma	JAK/STAT pathway regulator; member of the PIAS protein family
<i>eyes absent (eya)</i>	<i>Af</i> , Ma	Transcriptional cofactor regulating eye, gonad, brain development, axon pathfinding; immune response against DNA
<i>Clathrin light chain (clc)</i>	<i>Af</i> , Ma	Endocytosis
<i>CG11134</i>	<i>Af</i> , Ma	Methylthionibulose 1-phosphate dehydratase
<i>ADP ribosylation factor-like 2 (Arl2)</i>	<i>Af</i> , Ma	Small GTPase (ADP-ribosylation factor-like family); central regulator of microtubule growth and asymmetric division of neuroblasts
<i>CG7589</i>	<i>Af</i> , Ma	Orphan ligand-gated chloride channel; human ortholog = glycine receptor beta

C

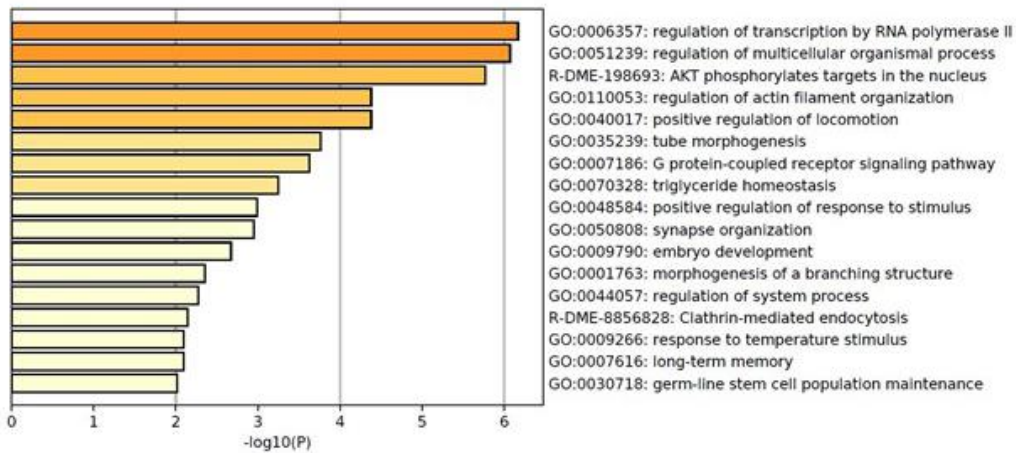
Gene name	Common hits	Gene function
<i>His2Av</i>	<i>Af</i> , VSV	Histone H2A variant
<i>CG17018</i>	<i>Af</i> , VSV	Meiosis regulator and mRNA stability factor 1
<i>mars</i>	<i>Af</i> , VSV	Microtubule-associated protein that shuttles between the mitotic spindle and the nucleus
<i>CG18265</i>	<i>Af</i> , VSV	uncharacterized protein
<i>Gapdh2</i>	<i>Af</i> , VSV	Glyceraldehyde 3 phosphate dehydrogenase 2
<i>CG15537</i>	<i>Af</i> , VSV	Human ortholog = Corticotropin-releasing factor-binding protein
<i>CG42709</i>	<i>Af</i> , VSV	uncharacterized protein; Leucine-rich repeat domain
<i>WRNexo</i>	<i>Af</i> , DCV	WRN exonuclease
<i>CG6230</i>	<i>Af</i> , DCV	uncharacterized protein; ATPase activity predicted
<i>schizo</i>	<i>Af</i> , DCV	Arf1 guanine nucleotide exchange factor involved in commissural axon guidance and myoblast fusion
<i>Sfp65A</i>	<i>Af</i> , DCV	Seminal fluid protein 65A
<i>CG1603</i>	<i>Af</i> , DCV	uncharacterized protein; DNA-binding, potential transcription cofactor
<i>CG11425</i>	<i>Af</i> , DCV	Phosphatidate phosphatase
<i>dare</i>	<i>Af</i> , DCV	Enzyme associated with steroid hormone synthesis and required for normal olfactory behavior
<i>Sos</i>	<i>Af</i> , DCV	Ras guanyl-nucleotide exchange factor activity
<i>Cdk9</i>	<i>Af</i> , DCV	Cyclin-dependent kinase 9; involved with CycT in RNA polymerase II elongation control
<i>Lkr</i>	<i>Af</i> , DCV	Leucokinin receptor; GPCR with the product of Lk as a ligand. It signals through intracellular calcium, and is implicated in ion regulation by the Malpighian tubules and in feeding control
<i>cap-n-collar</i>	<i>Af</i> , DCV	Nrf2; transcription factor that interacts with the product of Keap1 to regulate the activation of genes by oxidative stress
<i>bellwether</i>	<i>Af</i> , DCV	alpha subunit of the mitochondrial F1F0 ATP synthase complex (complex V), the final enzyme of the oxidative phosphorylation pathway
<i>CG4681</i>	<i>Af</i> , DCV	Human ortholog = TEX9 (testis-expressed 9)
<i>CG42355</i>	<i>Af</i> , DCV	Uncharacterized protein; High mobility group box domain superfamily
<i>Jhl-21</i>	<i>Af</i> , DCV	Juvenile hormone Inducible-21; L-amino acid transmembrane transporter activity
<i>CG3168</i>	<i>Af</i> , DCV	Major facilitator superfamily, sugar transporter-like; human ortholog = SV2A
<i>Mal-A7</i>	<i>Af</i> , DCV	Maltase A7
<i>CG11317</i>	<i>Af</i> , DCV	Zinc finger C2H2-type
<i>Hr4</i>	<i>Af</i> , DCV	Hormone receptor 4; nuclear receptor that acts both upstream and downstream of the steroid hormone ecdysone. In the prothoracic gland the product of Hr4 acts as a repressor of ecdysone production
<i>TfIIA-L</i>	<i>Af</i> , DCV	Transcription factor IIA L; precursor protein that, after proteolysis, produces the two larger subunits of TFIIA
<i>CG5880</i>	<i>Af</i> , DCV	Protein S-acyltransferase
<i>trx</i>	<i>Af</i> , DCV	Thioredoxin reductase-1
<i>Cdk12</i>	<i>Af</i> , DCV	Cyclin-dependent kinase 12
<i>Pendulin</i>	<i>Af</i> , DCV	Importin-alpha protein family, essential adaptor in the Ran-GTP nuclear transport cycle binding a cargo protein to the nuclear import receptor
<i>jim lovell</i>	<i>Af</i> , DCV	Transcription factor, DNA binding HTH domain, Psq-type
<i>Coop</i>	<i>Af</i> , DCV	Corepressor of Pangolin
<i>CG1504</i>	<i>Af</i> , DCV	BspA type Leucine rich repeat region
<i>mastermind</i>	<i>Af</i> , DCV	Transcriptional coactivator that functions in the Notch signaling pathway
<i>Rabphilin</i>	<i>Af</i> , DCV	Rab GTPase binding; human ortholog = RPH3A
<i>fork head</i>	<i>Af</i> , DCV	Direct target of Dpp/BMP signaling during midgut development; It also regulates antiviral gene expression
<i>CG7329</i>	<i>Af</i> , DCV	Uncharacterized protein; lipase activity
<i>tho2</i>	<i>Af</i> , DCV	Component of the THO complex that plays an essential role in nuclear export of mRNAs

Figure 6

A



B



C

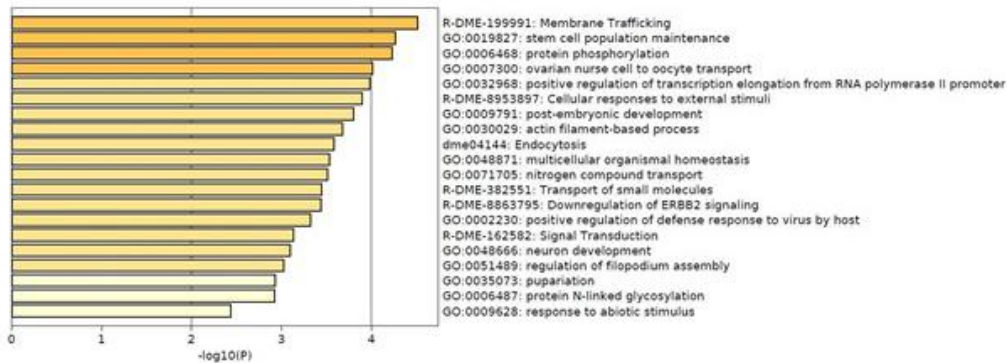


Table 1

		Virgin	Cross	Heating	Infection
2016	Aug21-Aug25	1st Pre-test			
	Aug28-Sep1		1st Pre-test		
	Sep4-Sep8				
	Sep11-Sep15	2nd Pre-test			
	Sep18-Sep22		2nd Pre-test		
	Sep25-Sep29			1st Pre-test	
	Oct2-Oct6	3rd Pre-test			1st Pre-test
	Oct9-Oct13		3rd Pre-test		
	Oct16-Oct20			2nd Pre-test	
	Oct23-Oct27	1st Primary test			2nd Pre-test
	Oct30-Nov3		1st Primary test		
	Nov6-Nov10			3rd Pre-test	
	Nov13-Nov17	2nd Primary test			3rd Pre-test
	Nov20-Nov24		2nd Primary test		
	Nov27-Dec1			1st Primary test	
	Dec4-Dec8	3rd Primary test			1st Primary test
	Dec11-Dec15		3rd Primary test		
	Dec18-Dec22			2nd Primary test	
	Dec25-Dec29	4th Primary test			2nd Primary test
	Dec26-Dec30		4th Primary test		
2017	Jan2-Jan6	1st retest		3rd Primary test	
	Jan9-Jan13		1st retest		3rd Primary test
	Jan16-Jan20				
	Jan23-Jan26			4th Primary test	
	Feb5-Feb9	Chinese new year	Chinese new year	Chinese new year	4th Primary test
	Feb13-Feb17	2nd retest		1st retest	
	Feb20-Feb24	5th Primary test	2nd retest		1st retest
	Feb27-Mar3		5th Primary test		
	Mar6-Mar10				
	Mar13-Mar17				
	Mar20-Mar24	3rd retest		2nd retest	
	Mar27-Mar31		3rd retest	5th Primary test	2nd retest
	Apr3-Apr7				5th Primary test
	Apr10-Apr14	6th Primary test			
	Apr17-Apr21		6th Primary test		
	Apr24-Apr28			3rd retest	
	May1-May5	7th Primary test			3rd retest
	May8-May12		7th Primary test		
	May15-May19			6th Primary test	
	May22-May26	8th Primary test			6th Primary test
	May29-Jun2		8th Primary test		
	Jun5-Jun9			7th Primary test	
	Jun12-Jun16	9th Primary test			7th Primary test
	Jun19-Jun23		9th Primary test		
	Jun26-Jun30			8th Primary test	

	Jul3-Jul7	10th Primary test			8th Primary test
	Jul10-Jul14		10th Primary test		
	Jul17-Jul21			9th Primary test	
	Jul24-Jul28	11th Primary test			9th Primary test
	Jul31-Aug4		11th Primary test		
	Aug7-Aug11			10th Primary test	
	Aug14-Aug18	12th Primary test			10th Primary test
	Aug21-Aug25		12th Primary test		
	Aug28-Sep1			11th Primary test	
	Sep4-Sep8	13th Primary test			11th Primary test
	Sep11-Sep15		13th Primary test		
	Sep18-Sep22			12th Primary test	
	Sep25-Sep29	14th Primary test			12th Primary test
	Oct2-Oct6		14th Primary test		
	Oct9-Oct13			13th Primary test	
	Oct16-Oct20	15th Primary test			13th Primary test
	Oct23-Oct27		15th Primary test		
	Oct30-Nov3			14th Primary test	
	Nov6-Nov10	4rd Retest			14th Primary test
	Nov13-Nov17		4rd Retest		
	Nov20-Nov24			15th Primary test	
	Nov27-Dec1	5th Retest			15th Primary test
	Dec4-Dec8		5th Retest		
	Dec11-Dec15			4rd Retest	
	Dec18-Dec22	Re-primary test			4rd Retest
	Dec25-Dec29		Re-primary test		
2018	Jan1-Jan5			5th Retest	
	Jan8-Jan12				5th Retest
	Jan15-Jan19				
	Jan22-Jan26	6th Retest		Re-primary test	
	Jan29-Feb2		6th Retest		Re-primary test
	Feb5-Feb9				
	Feb12-Feb16	chinese new year	chinese new year	chinese new year	chinese new year
	Feb19-Feb23	chinese new year	chinese new year	chinese new year	chinese new year
	Feb26-Mar2			6th Retest	
	Mar5-Mar9	7th Retest			6th Retest
	Mar12-Mar16		7th Retest		
	Mar19-Mar23				
	Mar26-Mar30	8th Retest			
	Apr2-Apr6		8th Retest		
	Apr9-Apr13			7th Retest	
	Apr16-Apr20	9th Retest			7th Retest
	Apr23-Apr27		9th Retest		
	Apr30-May4			8th Retest	
	May7-May11	10th Retest			8th Retest
	May14-May18		10th Retest		
	May21-May25			9th Retest	
	May28-Jun1	11th Retest			9th Retest
	Jun4-Jun8		11th Retest		
	Jun11-Jun15			10th Retest	
	Jun18-Jun22	12th Retest			10th Retest
	Jun25-Jun29		12th Retest		

	Jul2-Jul6			11th Retest	
	Jul9-Jul13	13th Retest			11th Retest
	Jul16-Jul20		13th Retest		
	Jul23-Jul27			12th Retest	
	Jul30-Aug3	14th Retest			12th Retest
	Aug6-Aug10		14th Retest		
	Aug13-Aug17			13th Retest	
	Aug20-Aug21	15th Retest			13th Retest
	Aug27-Aug31		15th Retest		
	Sep3-Sep7			14th Retest	
	Sep10-Sep14	16th Retest			14th Primary retest
	Sep17-Sep21		16th Retest		
	Sep24-Sep28			15th Retest	
	Oct1-Oct5	17th Retest			15th Retest
	Oct8-Oct12		17th Retest		
	Oct15-Oct19			16th Retest	
	Oct22-Oct26	1th New primary screen			16th Retest
	Oct29-Nov2		1th New primary screen		
	Nov5-Nov9			17th Retest	
	Nov12-Nov16	18th Retest			17th Retest
	Nov19-Nov23		18th Retest		
	Nov26-Nov30			1th New primary screen	
	Dec3-Dec7				1th New primary screen
	Dec10-Dec14				
	Dec17-Dec21			18th Retest	
	Dec24-Dec28				18th Retest
	Dec31-Jan4				
2019	Jan7-Jan11				
	Jan14-Jan18	2nd New primary screen			
	Jan21-Jan25		2nd New primary screen		
	Jan28-Feb1				
	Feb4-Feb8	chinese new year	chinese new year	chinese new year	chinese new year
	Feb11--Feb15	chinese new year	chinese new year	chinese new year	chinese new year
	Jan14-Jan23			2nd New primary screen	
	Jan14-Jan24				2nd New primary screen
	Jan14-Jan25				
	Jan14-Jan26				
	Feb25-Mar1	19th Retest			
	Mar4-8		19th Retest		

Table 2

	Primary test	Retest	Interesting hits
<i>A. fumigatus</i>	6471	1155	241

Table 3

TH number	CG number	gene name	TH number	CG number	gene name	TH number	CG number	gene name
THU4418	CG14168	Z band alternatively spliced PDZ-motif protein 67	THU2688	CG8843	Secretory 5	TH01561.N	CG15627	Ir25a
THU2173	CG1709	Vacuolar H+ ATPase 100kD subunit 1	TH01972.N	CG43748	H6-like-homeobox	THU5186	CG1057	MED31
THU3135	CG8795	Pyrokinin 2 receptor 2	THU1518	CG3585	Rabconnectin-3A	TH05162.N	CG5124	adipose
TH04955.N	CG8357	DNA fragmentation factor-related protein 1	TH04044.N	CG6016	bb in a boxcar	THU1052	CG43286	cnc
THU0955	CG17494	Sarcolemma associated protein	THU3521	CG7793	Son of sevenless	THU2444	CG18362	Mondo
THU0949	CG4303	Brahma associated protein 60kD	THU1068	CG8266	Secretory 31	THU2502	CG31256	Brf
THU3051	CG31646	Dpr-interacting protein θ	THU1106	CG8068	Su(var)2-10	THU3128	CG31235	
THU3052	CG34411	Leucine-rich repeat-containing G protein-coupled receptor 4	THU4047	CG4353	hemipterous	THU3153	CG10076	spire
THU5908	CG14884	COP9 signalosome subunit 5	THU3662	CG16778	jim lovell	THU3156	CG10382	wrappe
THU1639	CG6017	Huntingtin-interacting protein 14	THU2885	CG8118	mastermind	THU3167	CG1900	Rab40
THU3160	CG4910	Crustacean cardioactive peptide	TH02517.N	CG8544	scalloped	THU3188	CG12796	
THU1131	CG1483	Microtubule-associated protein 205	TH04668.N	CG6406	Hyccin	THU3192	CG16868	
THU1232	CG2903	Hepatocyte growth factor regulated tyrosine kinase substrate	THU2105	CG5295	brummer	TH01899.N	CG9701	
THU1508	CG13867	Mediator complex subunit 8	THU2079	CG10460	crammer	THU3479	CG5504	
THU3758	CG10260	Phosphatidylinositol 4-kinase III α	THU2473	CG33473	luna	THU1511	CG7508	atonal
THU2558	CG1864	Hormone receptor-like in 38	THU2480	CG7589		THU4098	CG15744	
THU2514	CG6103	Cyclic-AMP response element binding protein B	TH05256.N	CG6249	Cs14	THU0764	CG30388	Magi
THU5726	CG5896	Gram-positive Specific Serine protease	TH01657.N	CG4122	silver	THU2451	CG1603	
THU2255	CG3940	Carbonic anhydrase 7	TH02512.N	CG5125	ninaC	TH03476.N	CG9027	SOD 3

Table 4 & 5

TH number	CG number	gene name
THU2791	CG9901	Actin-related protein 2
THU2576	CG33135	KCNQ potassium channel
THU2495	CG43073	RIM-binding protein
THU0572	CG10539	Ribosomal protein S6 kinase
THU1837	CG1856	tramtrack

TH number	CG number	gene name
THU5328	CG6189	MYB binding protein 1a
THU1536	CG17697	frizzled
TH04555.N	CG18672	Carbonic anhydrase 16
THU2096	CG4822	
THU1887	CG42314	plasma membrane calcium ATPase
THU2078	CG42796	5-hydroxytryptamine (serotonin) receptor 2B
THU1386	CG15218	CycK
THU4955	CG7450	CrebA
THU0686	CG7597	Cdk12

Table 6

TH number	CG number	gene name	TH number	CG number	gene name	TH number	CG number	gene name
THU5725	CG15444		THU3838	CG9968	anxb11	TH04895 N	CG34404	
THU3940	CG8604	Amphiphysin	THU1725	CG17906		TH05000 N	CG7748	Catalytic subunit 3B of the oligosaccharyl transferase complex
THU4115	CG8786		TH01886 N	CG8839		TH05229 N	CG8668	
THU4044	CG5583	Ets at 98B	TH01896 N	CG6749		THU4011	CG1913	α -Tubulin at 84B
THU2291	CG43365	BTB-protein-VII	TH01898 N	CG6859	Peroxin 3	THU4009	CG45050	
THU0472	CG42355		TH02026 N	CG9092	Gal	THU4278	CG10376	
THU3173	CG7435	ADP ribosylation factor-like 2	TH04430 N	CG17018		THU5897	CG2368	pipspeak
THU0392	CG1504		TH04695 N	CG15863		THU0991	CG4609	failed axon connections
THU2185	CG8893	Glyceraldehyde 3 phosphate dehydrogenase 2	TH04821 N	CG15266	l(2)35Cc	THU4088	CG2124	
THU2900	CG3612	bellwether	TH05124 N	CG42479	Seminal fluid protein 65A	THU3936	CG11516	Protein tyrosine phosphatase 99A
THU0440	CG8269	Dynactin 2-p50 subunit	THU0751	CG32217	Su(Tpl)	THU4139	CG7259	Brestrophin 4
THU3000	CG32809		THU0909	CG4720	Apoptotic signal-regulating kinase 1	THU3778	CG8282	Sorting nexin 6
THU2121	CG11556	Rabphilin	THU3536	CG18262		THU4120	CG7749	kugelei
THU3473	CG10198	Nucleoporin 98-96kD	THU3625	CG8817	lilliputian	THU3905	CG8095	scab
TH04007 N	CG3790	Beta-alanine transporter	THU3865	CG5884	par-6	THU4117	CG17369	Vacuolar H ⁺ -ATPase 55kD subunit
THU1446	CG3469		THU5359	CG3312	RNA-binding protein 4F	THU1194	CG9916	Cyclophilin 1
THU3591	CG32697		THU5633	CG11880	Choline transporter-like 2	THU1251	CG14224	Ubiquilin
TH01684 N	CG16873	NimB5	THU3957	CG9496	Tetraspanin 29Fb	THU1267	CG17064	mars
THU1456	CG7700	Golgi SNARE, 28 kDa	THU3781	CG9779	Vacuolar protein sorting 24	THU3968	CG32434	schizo
THU2484	CG3168		TH03663 N	CG3059	NTPase defective in the avoidance of repellents	THU3992	CG7839	
THU3036	CG11134		THU3103	CG12390		THU1222	CG5899	Etl1
THU2778	CG4799	Pendulin	THU4004	CG8961	teflon	THU5045	CG17158	capping protein beta
THU2906	CG11317		TH03859 N	CG10263	Hakai	TH04945 N	CG4091	salivary glands marred
THU2705	CG1771	multiple edematous wings	TH04476 N	CG6726		THU2279	CG7345	Sox21a
THU0406	CG31671	tho2	TH03933 N	CG15552	Sox100B	THU1645	CG6521	Signal transducing adaptor molecule
THU0538	CG43934	Hormone receptor 4	THU3461	CG9667		TH04421 N	CG6610	
THU3096	CG34370		THU1483	CG3615	Autophagy-related 9	TH04422 N	CG7255	
THU3094	CG12239		THU1482	CG12334	Autophagy-related 8b	THU1885	CG11527	Tiggrin
THU3466	CG4143	multiprotein bridging factor 1	THU3477	CG42709		THU1873	CG1019	Muscle LIM protein at 84B
THU3987	CG8092	relative of voc	TH04689 N	CG7329		THU2658	CG6948	Clathrin light chain
THU3089	CG7234	Glutamate receptor IIB	TH04646 N	CG11777		THU3458	CG34422	hat-trick
THU3125	CG17739		THU0697	CG2910	spenito	THU2532	CG1621	Corepressor of Pangolin
THU1011	CG8651	trithorax	THU0649	CG12196	eggless	THU2545	CG10979	
TH02101 N	CG43128	Shaker cognate b	THU1620	CG12019	Cdc37	TH01673 N	CG2685	Splicing
TH01512 N	CG31729		TH04943 N	CG2082		THU1623	CG9347	neither inactivation nor afterpotential B
TH03750 N	CG11425		TH04925 N	CG10626	Leucokinin receptor	TH04439 N	CG8145	numerous disordered muscles
TH03692 N	CG4201	I-kappaB kinase β	THU0693	CG5499	Histone H2A variant	THU1154	CG12755	lethal (3) malignant blood neoplasm
TH03686 N	CG3738	Cyclin-dependent kinase subunit 30A	TH04939 N	CG32669		THU3811	CG7670	WRN exonuclease

TH number	CG number	gene name	TH number	CG number	gene name	TH number	CG number	gene name
THU0839	CG5745		THU4342	CG11423	NADH dehydrogenase (ubiquinone) 51 kDa subunit-like 1	THU3097	CG10137	
THU1618	CG3752	Aldh	THU4144	CG43946	glut1	THU3126	CG2016	
THU3501	CG6794	Dif	THU2570	CG2922	krasavietz	THU3121	CG15522	
THU4054	CG2679	gol	THU1125	CG11700		THU3133	CG7945	
TH01789N	CG4433		THU1294	CG9746	Vacuolar protein sorting 15	THU3169	CG31106	
TH02088N	CG14902	Decay	THU1375	CG14966		THU3178	CG18285	igloo
TH03073N	CG1316	CG1316	THU3469	CG18265		THU3186	CG11550	
TH03326N	CG4060	TwdIW	THU3216	CG13645	Nicotinamide mononucleotide adenylyltransferase	THU3187	CG12594	
TH03328N	CG4135	beat-IIb	THU3122	CG15537		THU1537	CG3619	Delta
TH03875N	CG6093	abo	THU2841	CG4944	ciboulot	THU3487	CG9556	alien
TH03971N	CG8967	otk	THU2884	CG7980	RabX5	THU1610	CG5179	Cyclin-dependent kinase 9
TH04230N	CG6230	CG6230	THU2789	CG8597	lark	THU3923	CG5798	Ubiquitin specific protease 8
TH04490N	CG5983	ACXC	THU2685	CG8548	karyopherin α 1	THU4223	CG12317	Juvenile hormone Inducible-21
TH04501N	CG11669	Mal-A7	THU3204	CG9245	Phosphatidylinositol synthase	TH03906N	CG5930	Transcription factor IIA L
TH04742N	CG13393	l(2)k12914	THU2446	CG10571	araucan	THU2077	CG4681	
THU0842	CG42799	dikar	THU2163	CG11988	neuralized	THU0453	CG8994	exuperantia
TH05273N	CG5880	CG5880	THU1923	CG1966	ATP-dependent chromatin assembly factor large subunit	THU5856	CG8669	cryptoccephal
TH04195N	CG18389	Eip93F	THU3058	CG12548	no mechanoreceptor potential B	THU5829	CG8049	Btk family kinase at 29A
THU4048	CG9554	eya	THU3055	CG15624	hoepel2	THU2460	CG10002	fork head
THU4228	CG6976	Myosin 28B1	THU1039	CG10155	Sprouty-related protein with EVH-1 domain	THU2434	CG5387	Cdk5alpha

Chapter 3 Insights of CycK/Cdk12-Nrf2 axis

Introduction

The expression of genes in higher eukaryotes is not regulated only at the initiation step. Indeed, many genes are poised for transcription and are actually paused at the beginning of the transcription unit through an interaction with the NELF complex (176). RNA polymerase 2 (PolII) elongation is also subject to regulations that are largely mediated through its C-terminal domain (CTD). The CTD is composed of dozens of a so-called heptad repeat YSPTSPS. When recruited to a gene, the PolII CTD is initially hypophosphorylated, then gets phosphorylated on Ser 5. Ser2 phosphorylation is required for productive elongation. Two complexes are required for the phosphorylation of Ser2 of the heptad repeats. The first one is composed of Cdk9 complexed to CycT and some models posit that this kinase works for transcription in the 5' moiety of the transcription unit. This phosphorylation step releases PolII from its paused state. CycK-Cdk12 would then further phosphorylate Ser2 during subsequent elongation (177, 178). However, it appears that while the action of the Cdk9/CycT complex is important for all transcripts, the Cdk12/CycK complex may be required in mammalian cultured cells for the production of genes with long transcripts and containing tens of exons (179). An initial study reported that several genes of the DNA repair pathway such as BRCA2 and FANCI are less expressed when the mammalian Cdk12/CycK complex is targeted by RNAi. Indeed,

the Cdk12/CycK complex is required for genomic stability (179, 180) and is involved in the development of cancers, and depending on the context, it may either act as an oncogene or as a tumor suppressor (181). An interesting study performed on *Drosophila* cultured cells aimed to identify through an RNAi approach the kinases that are involved in mediating the effects of the Nrf2 transcription factor, originally identified in flies as Cap and collar (Cnc), a gene encoding multiple isoforms (182). Cdk12 was the strongest hit of this limited screen (183). Interestingly, RNAi knock-down of CycK yielded a phenotype highly similar to that of Cdk12. Indeed, the complex is required for the expression of several validated transcriptional targets of Nrf2. Thus, there is a dual regulation of Nrf2 target genes: first, by the transcription factor itself at the initiation of transcription step, and second by the Cdk12/CycK complex at the elongation step. Of note, it is not clear whether the Cdk12/CycK complex is itself regulated nor how it recognizes its limited set of target genes (about 50 for activation and 150 for repression). As described further below, Nrf2 is the major mediator of the response to reactive oxygen species (ROS); the investigators analyzed by RNAseq gene expression in *Drosophila* cultured cells after a challenge with the strong oxidizer paraquat, in the presence or absence of the Cdk12/CycK complex. They identified 43 genes that encode proteins involved in antioxidant activity, including those with glutathione transferase or peroxidase activity, peroxiredoxins, or thioredoxin peroxidase activity (183). The study did not determine however whether the DNA repair pathway was affected in cells not treated with paraquat. Interestingly, we have performed an extensive RNAseq experiment at

different time points of infection by *M. anisopliae* under a natural infection or septic injury paradigm. We find that most of the genes identified by Li *et al.* are induced throughout the infection obtained through the injection of conidia but not during natural infection. Furthermore, genes involved in DNA repair do not appear to be induced during the infections.

Nrf2 (nuclear factor erythroid 2 related factor 2) is a bZIP transcription factor (184) that has been intensively investigated because of its major role in the response to oxidative stress (more than 1,000 references). It orchestrates the regulation of genes involved in cell cycle homeostasis, cytoprotection, innate immunity, and tumorigenesis. It also plays a role in inflammation-related disorders and Nrf2-null mice are highly susceptible to LPS-induced sepsis, an effect partially mediated by enhanced NF-kappaB and IRF3 signaling (185); Nrf2-binding sites have been found in the promoters of pro-inflammatory cytokines. Few studies have documented a role for Nrf2 during bacterial infections (186, 187). They tend to highlight that the innate immune response is enhanced in Nrf2 null mutant mice, which nevertheless succumb sooner to infection due to greater tissue damage. As regards viral infections, the situation is more contrasted and depends on the virus under consideration. Vesicular stomatitis virus is sensitive to ROS stress in macrophages (188, 189). ROS may also act indirectly by enhancing innate immune signaling (190). Herpes Simplex Virus 1 brain infections induce noxious ROS production by immune cells; stimulating the Nrf2 pathway by the plant compound sulforaphane alleviated neurotoxicity associated to ROS production by microglia cells (191). HIVs gp120 and Tat induce ROS in

endothelial cells and its associated toxicity can be mitigated by treatment with N-Acetyl-Cysteine amide (192). In alveolar macrophages of the lung, HIV infection through gp120 and Tat inhibits Nrf2 activity and thereby impairs their phagocytic function. Again, the induction of Nrf2 activity by sulforaphane treatment improved phagocytic function (193). The hepatic viruses HBV and HCV also induce oxidative stress through some of their regulatory proteins, resulting in the induction of some Nrf2 target genes. In the case of HBV, this results in the decreased production of the immunoproteasome, hence promoting viral proliferation by protecting the cell from ROS stress (194). HCV induced the Nrf2 pathway both through ROS-dependent and ROS-independent pathways that involve multiple signaling pathways including ERK, p38, PKC, PI3K, and casein kinase 2 (195). Furthermore, HCV promotes autophagy by triggering the ROS-induced phosphorylation of the autophagy adaptor p62. Phosphorylated p62 binds to KEAP1 and releases Nrf2, which is however kept inactive by being trapped to the ER by its binding partner Maf (see below). Again, this mechanism promotes viral proliferation (196). In summary, Nrf2 modulates rather negatively the innate immune response but promotes tissue repair, a resilience function. Viruses may hijack this system to their own advantage.

Mechanistically, Nrf2 is targeted for ubiquitin-mediated degradation by the proteasome by the KEAP1 cytoplasmic protein, which anchors it in the cytoplasm where Nrf2 is therefore present at low levels as long as KEAP1 is active. Upon exposure to an oxidative stress or to electrophiles, KEAP1 dissociates from Nrf2 that then binds to a small bZIP musculoaponeurotic fibrosarcoma protein Maf and

migrates to the nucleus to initiate transcription of its target genes, including KEAP1, which contain a consensus binding site in their promoters known as ARE (antioxidant response elements) (182). Nrf2 can also be regulated once in the nucleus through a GSK-3 β -dependent process that leads to its nuclear export (197). Of note, depending on the KEAP1 Cysteine residues targeted, at least five distinct categories of natural products (including sulforaphane) have been shown to trigger Nrf2 activation (198). Because KEAP1 is also forming a complex with PGAM5 and regulates a novel form of ROS-triggered, caspase independent cell death known as oxeiptosis (199), much promise is held by compounds that would specifically disrupt the interaction between KEAP1 and Nrf2, therefore stimulating anti-oxidant defenses. One must however remain aware that whereas ROS at high doses are detrimental by affecting lipids, proteins and nucleic acid, many physiological processes depend on signaling mediated by low levels of ROS, including NF-kappaB signaling.

In the course of the large-scale RNAi screen based on monitoring the survival to an injection of some 250 *Aspergillus fumigatus* conidia, one of the first hits we obtained was *CycK*, even though its phenotype was not thoroughly established as the mutant is semi-lethal. Here, I report my investigations on this gene and its potential function in mediating the Nrf2-dependent response to ROS. An open question is whether ROS are generated during the course of the *A. fumigatus* infection.

Results

Susceptibility to A. fumigatus infections

We have found that flies in which the CycK gene is targeted by a RNAi transgene at the adult stage rapidly succumb to *A. fumigatus* infection, as fast as the Toll pathway mutant *MyD88* (**Fig. 1A**). In retests, it became apparent that CycK flies that have been injected with PBS + 0.01% Tween20 as a control also succumb, like noninfected mutants, but at a slower rate than infected flies. Thus, this gene is also required for the viability of adult flies. An enhanced mortality has also been detected using *M. anisopliae* or DCV in the parallel screens led at the Sino-French Hofmann Institute. In these cases however, it is difficult to determine whether this increased susceptibility to infection reflects a *bona fide* effect or results just from the addition of the infection to that of the frailty of the CycK mutants. Interestingly, the *A. fumigatus* fungal load did not increase in these mutants, as is the case for *MyD88* (**Fig. 1B-C**). Thus, our preliminary data suggest that CycK may be involved in resilience to at least some fungal and viral infections. CycK functions in a complex with the Cdk12 kinase. Cdk12 has been tested in the primary screen and does present a similar phenotype of enhanced susceptibility to *A. fumigatus* or VSV infection. One preliminary retest experiment suggests that the Cdk12 and Nrf2 mutant is also sensitive to *A. fumigatus* infection (**Fig. 2, 3**). Of note, in both cases the uninfected and buffer injection controls did not succumb at all. Thus, it is unlikely that the phenotype we observe is due to an off-target effect of the shRNA transgene.

Susceptibility to mycotoxins

In a preliminary series of experiments, we found all the CycK, Cdk12, and Nrf2 mutant flies to be sensitive to DMSO, thus making the interpretation of any mycotoxin dissolved in pure DMSO difficult. We used the only toxin, restrictocin, which is soluble in an aqueous environment, namely PBST, to test the mutant flies. Only the *Cdk12* RNAi mutant displayed a clear cut sensitivity phenotype, although it was somewhat delayed (**Fig. 5**). For CycK and Nrf2, we still need to do a dose response test to find the optimal concentration to perform a relevant survival experiment to be able to conclude on their sensitivity to restrictocin (**Fig. 4, 6**).

Discussion

As described in chapter 1, we are just beginning to study resilience in animals. In *Drosophila*, besides studies on intestinal host defenses, little is known in the context of systemic infections (200, 201). Indeed, a couple of genetic approaches performed using a natural infection paradigm by *M. anisopliae* identified only one line that appeared to have a lessened resilience (162). One of the reasons for implementing the large-scale genetic screens was to identify lines displaying an altered resilience. As mentioned in chapter 2, we have so far identified one such gene in our *A. fumigatus* screen, *CrebA*, which has just been reported to be involved in resilience to bacterial infections (174). This gene was however not picked up in our *M. anisopliae* natural infection survival screen and it is an open possibility that this transcription factor plays a role in host defense only against pathogens introduced through a septic wound. Our preliminary data suggest that the CycK/Cdk12 genes are likely involved in resilience to at least *A. fumigatus* and VSV infections. According to these data, our hypothesis is that ROS are generated during the infection and that the CycK/Cdk12 complex will be required to mediate an anti-oxidant resilience response that will limit the damages exerted directly by the pathogen virulence factors or more likely indirectly via the host's own immune response. We further suppose that Nrf2 is also involved in this response by initiating the transcription of genes with anti-oxidant activities while the CycK/Cdk12 genes would act as described by controlling the transcription elongation of Nrf2-regulated genes as well as other genes that remain to

be identified.

When assessing the literature on ROS and infections, it appears that most of the work has been performed in cell culture models with an emphasis on the role of the ROS burst upon phagocytosis of bacteria or fungi. In this case, the link with the physiology of the whole organism has hardly been studied. As regards viruses, the situation appears to be highly dependent on the virus under study. It is striking that even though several studies the existence of a ROS response to viral infections, the molecules involved in generating this ROS burst have mostly not been identified. It is thus not clear how cells cope with a ROS exposure that is poorly delineated. While the antioxidant response and the role of Nrf2 has attracted considerable attention, there is hardly any specific emphasis to understand how it plays a role in resilience at the level of the organism during infections. A link between CycK/ Cdk12 and Nrf2 has only been established in *Drosophila* cell culture and validated to some extent *in vivo* by considering the response to ROS exposure and not to infection (183). We aim in the future to use the full power of the *Drosophila* model organism to study in detail these issues. A strong asset is the ability to test rapidly several conditions and to move easily from the level of the cell or tissue to that of the whole organism. Thus, one major original point is the establishment of a link between CycK/Cdk12 and Nrf2 in the context of infection. A second outstanding merit of the project is the parallel investigation of a fungal and a viral pathogen. Finally, the combination of reporter transgenes for ROS or antioxidant genes and functional approaches by tissue-specific RNAi will provide an unprecedented glimpse on how ROS exposure arises during

infections at the whole organism level. In addition, our genetic tools should allow us to define how oxidative conditions are triggered during viral infections.

In *Drosophila*, Nrf2 has a second function. It appears to be a major regulator of the detoxification response induced by exposure to xenobiotics (202). It is not known whether the CycK/Cdk12 complex is also involved in this second function. Our preliminary experiments yield somewhat disparate results: Nrf2 does not seem to be sensitive to the action of restrictocin; this result needs to be reproduced while insuring that there is indeed an Nrf2 phenotype that should be assessed in parallel: induction of some specific cytochrome P450 genes after exposure to xenobiotics and the most important, susceptibility to *A. fumigatus*. The *CycK* data cannot be interpreted as the mutant lines were too sensitive to buffer injection in this experiment. Finally, the enhanced sensitivity of *Cdk12* RNAi mutants needs to be confirmed.

Material and methods

All the material and methods are the same to the corresponding parts in chapter 1 and 2.

Figure Legends

Figure 1: Infection traits of Ubi Gal4Gal80>CycK^{RNAi} mutant flies

A. Survival of Ubi Gal4Gal80>CycK^{RNAi} mutant flies after the injection of 250 Af conidia per fly; there is a significant difference between not injected (NI) and PBST-injected control (PBST) on the one hand and *A. fumigatus* infected (Af) Ubi Gal4Gal80>CycK^{RNAi} flies on the other ($P < 0.0001$). Data were analysed using log-rank test.

B. Fungal load in different lines after the injection of 250 Af conidia per fly.

C. Fungal load upon death in MyD88 and Ubi Gal4Gal80>CycK^{RNAi} mutant flies, Af 250 conidia/fly injection, there is significant difference between 0h and FLUD in MyD88 ($P < 0.0001$), but not in Ubi Gal4Gal80>CycK^{RNAi} ($P = 0.3375$). Data were analysed using Mann-Whitney test.

Figure 2: Survival of Ubi Gal4Gal80>Cdk12^{RNAi} mutant flies in retest

Survival of Ubi Gal4Gal80>Cdk12^{RNAi} mutant flies after the injection of Af 250 conidia per fly; there is a significant difference between not injected (NI) and PBST-injected control (PBST) on the one hand and *A. fumigatus* infected (Af) Ubi Gal4Gal80>Cdk12^{RNAi} flies on the other ($P < 0.0001$). Data were analysed using log-rank test.

Figure 3: Survival of Ubi Gal4Gal80>Nrf2^{RNAi} mutant flies in retest

Survival of Ubi Gal4Gal80>Nrf2^{RNAi} mutant flies after the injection of Af 250 conidia per fly; there is a significant difference between not injected (NI) and PBST-injected

control (PBST) on the one hand and *A. fumigatus* infected (Af) Ubi Gal4Gal80>Nrf2^{RNAi} flies on the other ($P < 0.0001$). Data were analysed using log-rank test.

Figure 4: Survival of Ubi Gal4Gal80>CycK^{RNAi} mutant flies after the injection of restrictocin

Survival of Ubi Gal4Gal80>CycK^{RNAi} mutant flies after the injection of 1 mg/ml, 4.6 nl per fly. There is no significant difference between PBST-injected control (PBST) and restrictocin-injected (R) Ubi Gal4Gal80>CycK^{RNAi} flies ($P = 0.1100$). Data were analysed using log-rank test.

Figure 5: Survival of Ubi Gal4Gal80>Cdk12^{RNAi} mutant flies after the injection of restrictocin

Survival of Ubi Gal4Gal80>Cdk12^{RNAi} mutant flies after the injection of 1 mg/ml, 4.6 nl per fly. There is a significant difference between PBST-injected control (PBST) and restrictocin-injected (R) Ubi Gal4Gal80>Cdk12^{RNAi} flies ($P = 0.0003$). Data were analysed using log-rank test.

Figure 6: Survival of Ubi Gal4Gal80>Nrf2^{RNAi} mutant flies after the injection of restrictocin

Survival of Ubi Gal4Gal80>Nrf2^{RNAi} mutant flies after the injection of 1 mg/ml, 4.6 nl per fly. There is no significant difference between PBST-injected control (PBST) and restrictocin-injected (R) Ubi Gal4Gal80>Nrf2^{RNAi} flies ($P = 0.4386$). Data were analysed using log-rank test.

Figure 1

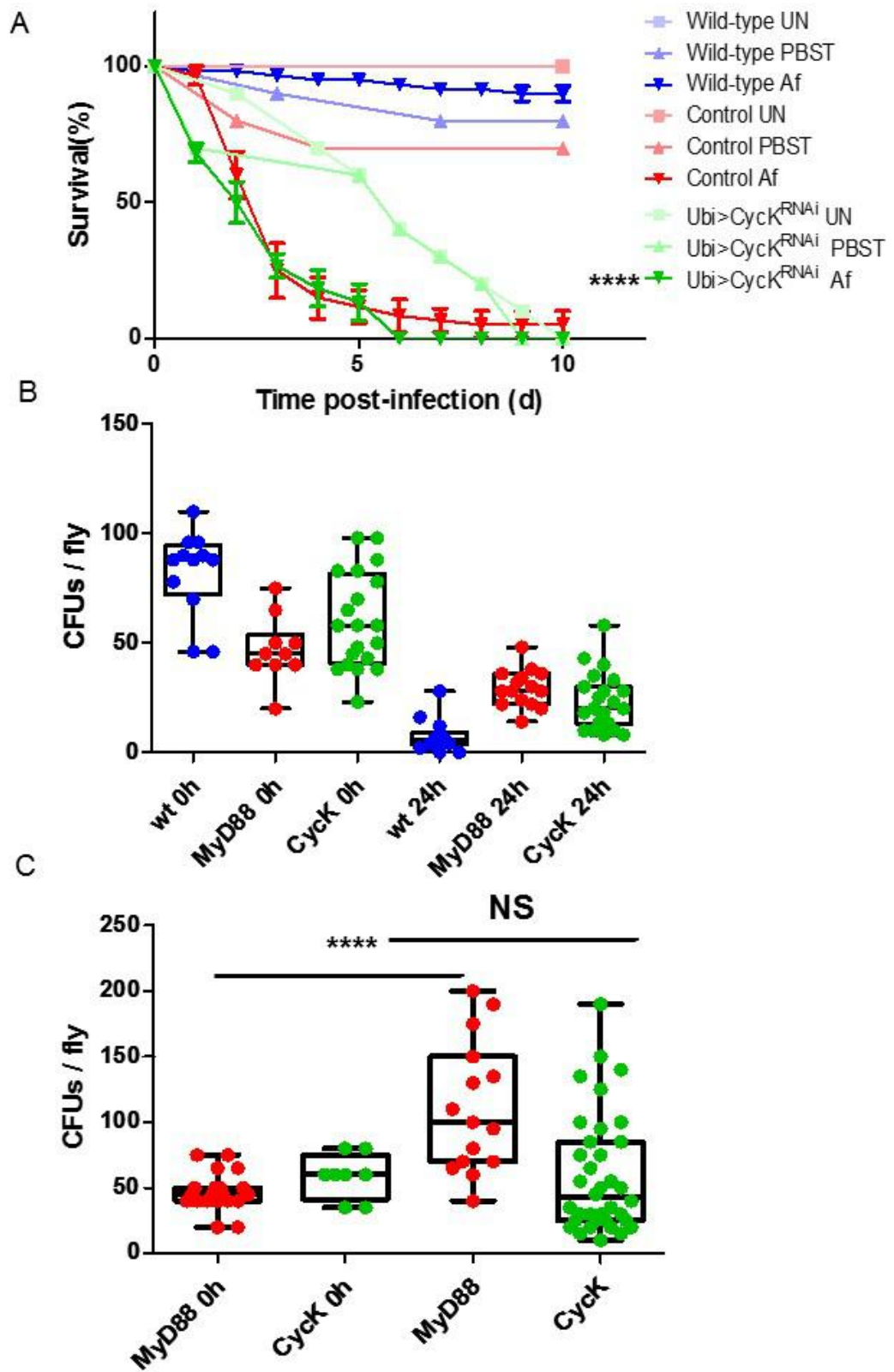


Figure 2

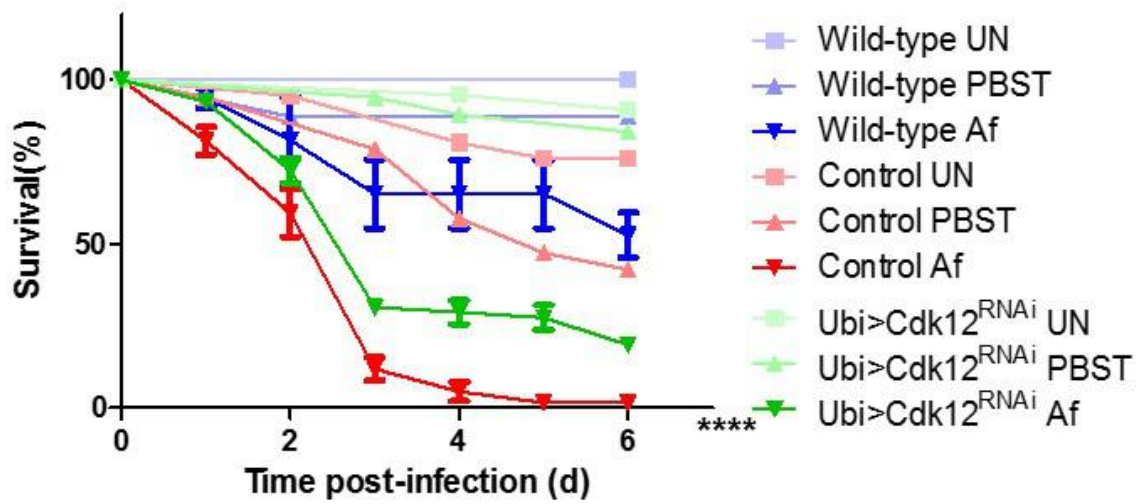


Figure 3

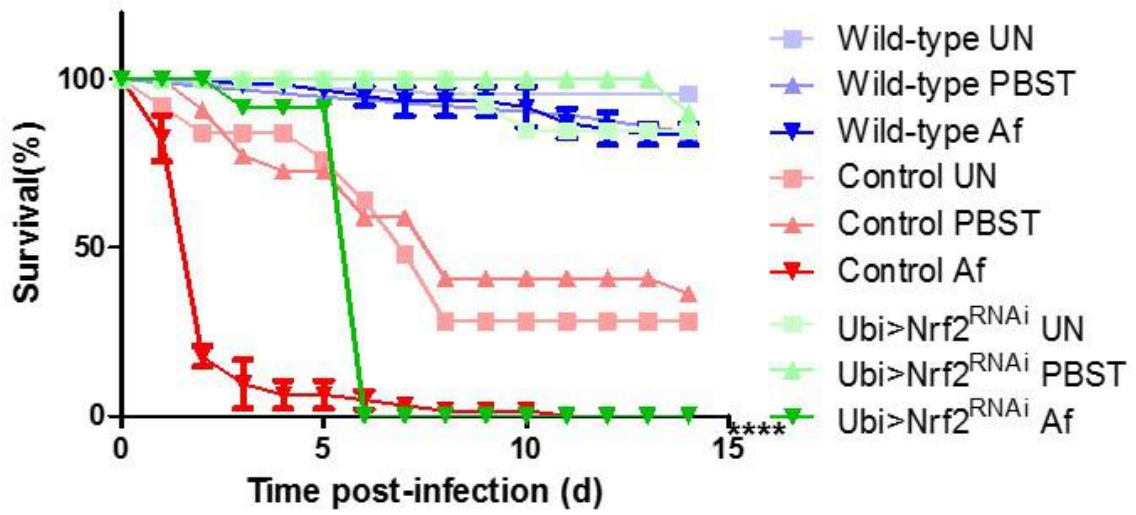


Figure 4

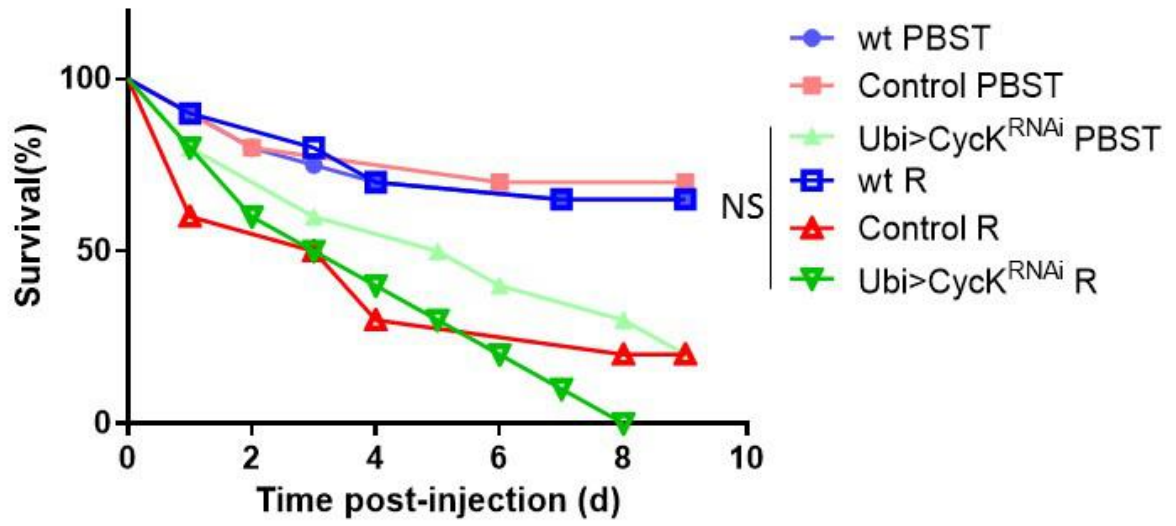


Figure 5

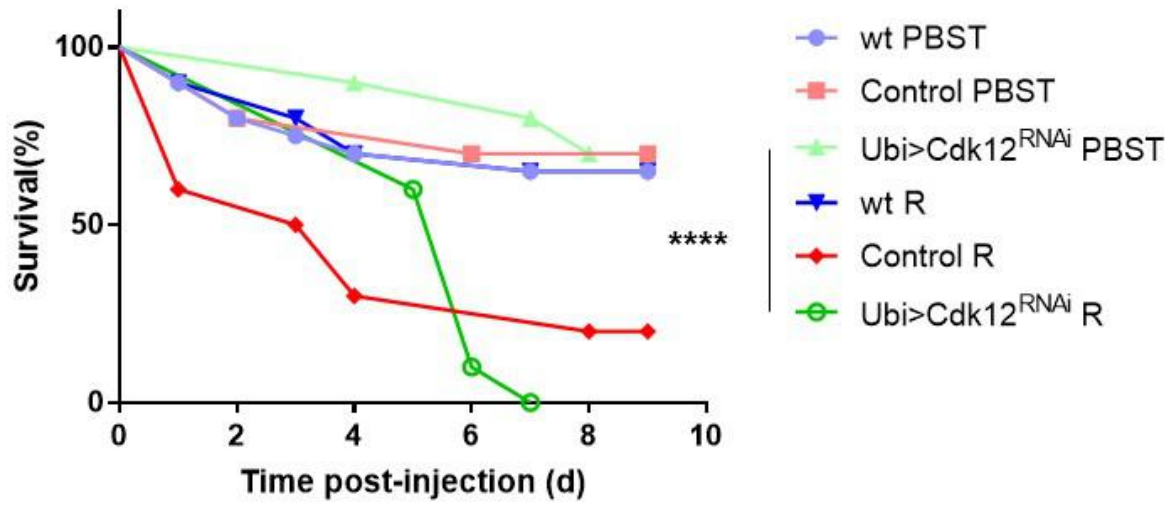
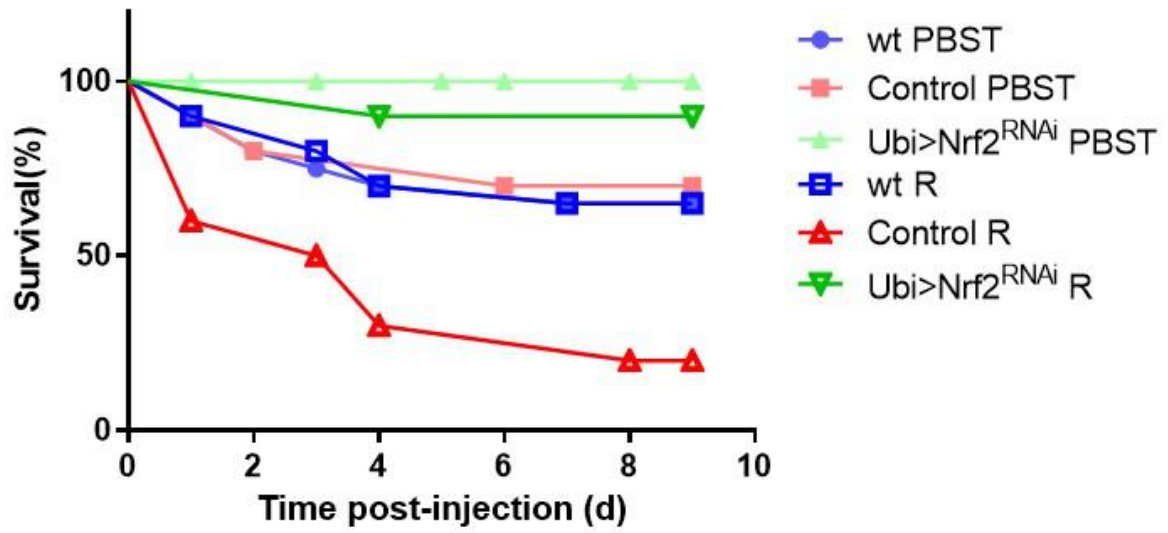


Figure 6



Chapter conclusions

The goal of this thesis was to advance our current understanding of fungal infections, and especially those mediated by *A. fumigatus*. The initial expectation was that the large-scale genetic screen would yield enough interesting mutants rapidly enough to focus on some of them for detailed study. However, an initial step was first to re-establish carefully a valid infection model and then to characterize it in detail. We then made an unexpected finding discussed more in detail below and decided to focus on it as this observation has the potential to cause a paradigm shift in the manner we understand and study *A. fumigatus* infection.

In the first part, we demonstrated that *MyD88* does not play a resistance as expected but more a resilience role in the host defense to against *A. fumigatus* infection. Indeed, the overexpression of the most strongly expressed antifungal peptide active on hyphae, Drosomycin, did only modestly provide some protection to *Spätzle-imd* double mutant flies under conditions of likely high inoculum load (145). A recent study reported that flies devoid of most known AMPs, including the antifungal peptides Drosomycin and Metchnikowin, and cecropins, were only slightly more susceptible to a high inoculum of *A. fumigatus* (163). Thus, even under these condition of high initial fungal burden (likely around 20,000 spores per fly according to Lionakis *et al.* (167), it is likely that host defense against *A. fumigatus* does not rely solely on the effect of the known antimicrobial peptides but involves other molecules, possibly unidentified or uncharacterized antifungal peptides such as DIM genes first identified

through mass-spectrometry analysis or alternatively and nonexclusively a mechanism akin to that revealed in this work. Under our low inoculum conditions, the Toll pathway does not appear to be required to limit the proliferation of the invading fungus. Indeed, a dose of 50 injected conidia suffices to kill *MyD88* flies yet does not trigger a detectable activation of the Toll pathway in wild-type flies. In contrast, melanization appears to be important to prevent the dissemination of the fungus. Strikingly, *Hayan* melanization-deficient flies, even though they harbor a higher fungal load upon death, are nevertheless much more resistant to *A. fumigatus* than *MyD88* flies. It follows that the control of the dissemination of the fungus is not a critical parameter of the host defense against this infection. It is however perplexing that *PPO2* mutants display a more severe phenotype than that of *Hayan*. This issue deserves more intensive investigations and may reflect a requirement for another protease to fully mature PPO2 into active PO2. It will be especially interesting to determine the fungal proliferation in the *PPO2* mutant, as compared to that of *Hayan*. We suspect that in this mutant the fungal load might be much higher than for *Hayan*, in which the fungal burden remains relatively low even though it is higher than in *MyD88* mutants. It would then be interesting to determine whether the immune response is effective against *A. fumigatus in vivo*, possibly by overexpressing AMPs in *PPO2* mutants.

Our data suggest that the ability to cope with the exposure to mycotoxin is the relevant host defense present in wild-type and *hayan* flies, but lacking in *MyD88* flies, even when the Toll pathway does not appear to be stimulated in the case of a low

inoculum dose. Furthermore, the injection of toxins did not trigger an increased expression of *Drosomycin*. Our finding that an *A. fumigatus* strain devoid of secondary metabolism is less virulent supports this hypothesis, even though the restrictocin protein is still expected to be produced in this strain. Our current approach allows us to determine which toxin is sufficient to kill *MyD88* flies but fails to address the question of which one is critically required or whether there is some degree of redundancy between these toxins. A more careful analysis will be required and involve fungal mutants in which the synthesis of one toxin or family of toxins is ablated at a time. It is an open possibility that a combination of such mutations may be required to abolish the virulence of *A. fumigatus* in the fly in the case of partial redundancy. An important goal will be to discriminate between the functions of toxins that may target the nervous system from those such as restrictocin that may work ubiquitously in any cell type of the fly since their targets are expressed in all cells. This approach from the fungal side will require an expertise in the microbiology of *A. fumigatus* that is unavailable at present. One strategy will be to collaborate with a laboratory interested in this issue or alternatively, we shall have to generate such strains ourselves after adequate training. We note that some mutants have already been generated, for instance affecting the verruculogen/fumitremorgin synthesis pathway but are encountering adverse conditions as the authors of that study have so far failed to reply positively to our multiple requests. Possibly, metabolomics approach might have to be implemented to detect the secreted mycotoxins in the hemolymph or tissues of the host. It may actually be worth attempting hemolymph

transfer experiments from an infected host nearing its demise to a naive *MyD88* host, although the transferred quantities are likely to be insufficient to detect some toxicity. In the future, several venues of investigation from the host side need to be implemented. A pressing issue is to determine whether the resilience to mycotoxins phenotype is indeed due to the *MyD88* mutation, and next whether it involves the whole or only part of the Toll pathway. These studies will be important to delineate how this pathway is involved. We note that most studies on the extracellular arms of the pathway that lead to the processing of Spätzle through proteolytic cascade activation did not use *A. fumigatus* but usually other fungi or Gram-positive bacteria to analyze their mutant phenotypes. Thus, the careful characterization of *A. fumigatus* infection needs to be repeated for the other members of the pathway, an undertaking currently underway. A major question to be answered is whether there is an involvement of the canonical Toll pathway. Besides the genetic approach, transcriptomics using our low *A. fumigatus* inoculum at doses that induce or not the Toll pathway and a comparison to the injection of toxins will need to be implemented, as it is likely that our usual readouts are not relevant in this context. It will also be important to determine whether the host xenobiotics detoxification pathways are also required for the resilience of wild-type flies to mycotoxins. Nrf2 is a transcription factor mediating most of the detoxification response and has been picked up in our screen. As discussed further below, it may display this phenotype because of its function in the response to oxidative stress. Our preliminary trials with the injection of restrictocin have yielded a rather confusing picture at present. We shall keep in mind

that the proposed Nrf2/CycK/Cdk12 axis thought to be active in the host defense against ROS may not work in a similar manner in response to *A. fumigatus* or mycotoxins.

In the large-scale screen, we initially refined the strategy then performed it under optimized high-quality conditions. It involves a considerable organization and amount of work and we have so far tested about half of the “classical” coding genome, which comprises some 13,000 protein coding genes. We would like to screen all the available Trip lines, the barrier to obtaining these lines being administrative. One striking result is that flies with an apparent intact immune system as we understand it today are sensitive to *A. fumigatus* infection. They may affect resilience to infection rather than resistance, although this is difficult to establish formally. Indeed, the assay used to diagnostic a resilience function is that flies should succumb in the absence of an increased microbial burden. As this is already the case for *MyD88* immunodeficient flies, this criterion cannot readily be used. We shall need to rely on more indirect criteria, that is, to exclude the possibility that the immune defenses of the mutant flies are not affected, that is, have a normal induction and secretion of AMPs, a normal cellular and melanization response. At present, most of our putative candidates are not known to affect the immune response and therefore constitute interesting leads to understand resilience. It is important to emphasize at this point that we are still far away from having formally confirmed these mutants and therefore we have to be very cautious in interpreting our findings. This is one reason why we did not delve too much on the function of our present hits a it is somewhat premature.

It is nevertheless clear that mutations that affect genes involved in nervous system function represent attractive candidates to investigate the resilience to the tremorgenic toxins. One roadblock is that three of the toxins need to be resuspended in a toxic buffer, DMSO. After initial solubilization in DMSO at high concentration, our attempts at diluting further in an aqueous buffer seemingly lead to the precipitation of part or the whole of the compound, thereby affecting the rigorous estimation of the injected doses. We shall try to find secondary solvents that are less toxic to the flies. We inject 4.6 nl per fly when the amount of hemolymph is estimated to be of about 100 nl.

The identification of the sensitivity to infection of *Nrf2*, *CycK*, and *Cdk12* RNAi mutants open the possibility of having this axis functioning in host defense against fungi and at least one virus. Besides a potential role in the detoxification of mycotoxins, the other possibility is that of an involvement in the response to oxidative stress, which is more in keeping with the fact that these mutants have also been picked up in the DCV screen, and *CycK* in the *M. anisopliae* screen. DCV is unlikely to work through toxins, although it might be pathogenic especially in some tissue such as the nervous system. We are however unaware that it proliferates there although a detection of the virus in some neurons of the nerve chord cannot be excluded. It will thus be important to determine whether ROS are produced during viral and fungal infections and whether they play a role. This represents a full research project on its own in as much as ROS are notoriously difficult to visualize reliably in the fly. The use of reporter transgenes for different ROS or for the transcriptional activity of Nrf2

will have to be complemented by biochemical approaches. For instance, the sensitivity phenotype shall be tested for a possible rescue by exposure to reducing agents or the overexpression of catalases or peroxidases. As a side note, it will be interesting to ask whether the hits in common with the DCV screen also have an antiviral function in cultured S2 cells, a procedure implemented in the group of *Prof. Peng*.

In conclusion, the major contributions of this work partially fulfill my initial goals. On the one hand, I have initiated a large-scale screen that identifies interesting candidate genes for further study. This screen can be further pursued provided the access to mutant lines is solved. On the other hand, the finding of the overwhelming importance of the resilience to mycotoxins in *Drosophila* host defense opens novel directions to understand the pathogenicity of this pathogen. While the importance of mycotoxins in *A. fumigatus* virulence has been documented for a long time, with mycotoxins detected in fluids of infected patients, it remains unclear to this day how the organism is able to cope with this toxin. Hopefully our future findings in *Drosophila* will also be relevant to an understanding of our own defenses against *A. fumigatus* mycotoxins.

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UNIVERSITE DE STRASBOURG

RESUME DE LA THESE DE DOCTORAT

Discipline : Biologie Moléculaire et Cellulaire

Spécialité : étude des relations hôte-pathogène

Présentée par : XU Rui

(Nom-Prénom du candidat)

Titre : Vers une meilleure compréhension de la résistance et de la résilience dans la défense de l'hôte contre le champignon pathogène opportuniste *Aspergillus fumigatus* chez *Drosophila melanogaster*

Unité de Recherche :

- 1) UPR 9022 du CNRS : Modèles insectes de l'immunité innée Strasbourg
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Les infections fongiques constituent encore de nos jours une des causes majeures de mortalité due aux maladies infectieuses. En effet, les champignons font environ 1,6 millions de victimes dans le monde chaque année, plus que le SIDA, la tuberculose ou les pneumonies. Néanmoins, leur étude est beaucoup moins développée que celle des autres maladies sus-citées. L'ascomycète *Aspergillus fumigatus* est un pathogène opportuniste particulièrement dangereux et à l'origine d'une morbidité et d'une mortalité importante, 50% des patients succombant à l'infection malgré un traitement médical souvent administré trop tardivement car non détecté initialement. Un des atouts essentiels d'*A. fumigatus* par rapport à la majorité des espèces de champignon est sa capacité à supporter des températures élevées, au-dessus de 50°C, températures parfois atteintes dans les composts. Il est ubiquitaire et présent dans l'air à raison de 50 spores par m³ dans un environnement non contaminé. La petite taille de ses spores, les conidies, lui permet de pénétrer au plus profond de nos voies respiratoires. Les neutrophiles sont particulièrement efficaces pour prévenir un passage dans la circulation générale et il est aisé de concevoir la menace qu'il constitue en cas de neutropénie, par exemple suite à une chimiothérapie. *A. fumigatus* est aussi capable d'infecter d'autres organes comme l'oeil, le tractus gastro-intestinal, la peau et les ongles, et le système nerveux central. Ce champignon filamenteux se développe essentiellement par un cycle asexuel et les conidies germent pour former un mycélium. Le champignon est détecté par le système immunitaire inné et secondairement par le système immunitaire adaptatif; notamment, un composé de sa paroi, le $\beta(1, 3)$ glucane active le récepteur dectin-1; ces composés sont toutefois masqués par des hydrophobines. Parmi les facteurs de virulence identifiés figurent des toxines émises par *A. fumigatus*, dont la plus connue est peut-être la gliotoxine, laquelle est capable d'inhiber la voie majeure de l'inflammation, la voie NF-kappaB^{1,2}.

La mouche du vinaigre *Drosophila melanogaster* constitue un modèle d'étude très puissant, en particulier en raison de sa génétique sophistiquée développée depuis plus d'un siècle. Son système immunitaire est relativement bien étudié. Ainsi, trois types de réponses sont déclenchés suite à une blessure septique³. La première est relayée par

le déclenchement de cascades de protéases qui aboutissent à l'activation d'une ou plusieurs phénol-oxydases, lesquelles sont requises pour le dépôt de mélanine au site de blessure et pourraient générer des espèces oxygénées réactives et radicaux libres qui pourraient aussi agir sur les microbes introduits au niveau de la blessure. Une deuxième réponse est cellulaire et implique la phagocytose des microorganismes par les hémocytes de la drosophile. La troisième est la réponse humorale systémique laquelle implique deux voies régulatrices de type NF-kappaB⁴. Alors que la voie Immune deficiency est déclenchée par des bactéries à Gram-négatif et des bacilles dont la paroi comprend du peptidoglycane de type di-amino-pimélique, la voie Toll quant à elle est préférentiellement induite par des infections fongiques et des infections bactériennes d'espèces dont le peptidoglycane est de type Lysine. De manière générale, chaque voie est efficace contre les pathogènes qui la déclenche, à l'exception de certains pathogènes résistants aux principaux médiateurs de la réponse humorale, les peptides antimicrobiens. D'autres pathogènes pourraient interférer avec la réponse NF-kappaB, voire la bloquer. Une des particularités de la voie Toll est qu'elle est déclenchée par des récepteurs circulants qui détectent soit les $\beta(1,3)$ glucanes des parois fongiques soit le peptidoglycane de type Lys⁵⁻⁷. Ils initient alors des cascades protéolytiques qui aboutissent à activer par clivage le ligand Spätzle (homologue des neurotrophines humaines) du récepteur Toll. Une deuxième cascade de protéase est quant-à-elle déclenchée par les activités protéolytiques de facteurs de virulence sécrétés par des pathogènes fongiques ou bactériens⁷⁻⁹. Les voies IMD et Toll aboutissent chacune à l'expression d'un éventail spécifique de gènes codant des peptides antimicrobiens. Ainsi, la Drosomycine dont l'expression est activée par la voie Toll agit sur certains champignons filamenteux et aboutit à leur lyse, ce qui a pu être confirmé *in vivo*^{10,11}. Par ailleurs, d'autres peptides dont les gènes se retrouvent dans la région 55C du génome sont actifs contre une variété de pathogènes, y compris *Candida glabrata*, une levure pathogénique^{12,13}. Celle-ci ne prolifère pas et ne tue pas les drosophiles sauvages. Au contraire, elle se multiplie dans les mouches déficientes pour l'activation de la voie Toll¹⁴. En aboutissant au contrôle de la prolifération de certains pathogènes, voire leur lyse, la voie Toll apparaît donc comme une voie de

résistance de la défense de l'hôte contre les infections fongiques. La **résistance** est une des deux dimensions de la défense de l'hôte contre les infections et aboutit généralement à la neutralisation ou à l'annihilation des pathogènes: elle correspond à la réponse immunitaire. Cependant, une deuxième dimension de la défense de l'hôte contre les infections existe et a été nettement moins étudiée: la **résilience** correspond à la capacité de l'hôte à endurer et à réparer les dommages occasionnés par l'infection, soit suite à l'action des facteurs de virulence du pathogène, soit infligés par la propre réponse immunitaire de l'hôte¹⁵. Cette deuxième dimension de la réponse immunitaire n'a presque pas été étudiée dans le cas des infections fongiques.

L'équipe animée par le Pr. Dominique Ferrandon au sein du Sino-French Hoffmann Institute de la Guangzhou Medical University approche les infections fongiques chez la drosophile de manière globale, d'une part à l'aide de mutagénèses relativement peu biaisées car le paramètre suivi est la survie à l'infection fongique, et d'autre part en étudiant la voie Toll et le rôle des gènes régulés par cette voie dans la défense de l'hôte contre les infections fongiques ou bactériennes. En ce qui concerne les infections fongiques, un premier modèle est le champignon entomopathogénique *Metarhizium anisopliae*, lequel tue les drosophiles soit dans un modèle d'infection par injection soit en traversant la cuticule après dépôt des spores dessus. Compte tenu de mon parcours médical, j'ai quant à moi décidé de développer un autre modèle d'infection, avec le champignon opportuniste *A. fumigatus*. Celui-ci avait été utilisé comme illustration du rôle antifongique de la voie Toll dans la publication princeps du laboratoire CNRS dirigé par Jules Hoffman à Strasbourg¹⁶. Cependant, peu d'études sur *A. fumigatus* dans ce modèle ont été conduites par la suite¹⁷. Il a pu toutefois être établi que la surexpression ectopique de la Drosomycine protège faiblement les mutants *Spätzle* contre cette infection¹¹. De même, un mutant dans lequel les principaux gènes codant des peptides antimicrobiens sont délétés ne montre qu'une susceptibilité modeste à l'infection. Pour ma part, j'ai établi un modèle d'infection par injection et caractérisé en détail ce modèle, ce qui m'a conduit à une découverte inattendue; en parallèle, j'ai mis en place et commencé un large crible génétique afin d'identifier des souches mutantes présentant une susceptibilité accrue à

A. fumigatus. Ce crible a été poursuivi en Chine par une autre doctorante, Mme Chuqin Huang, lorsque je suis venu en France à Strasbourg pour une année de recherche où je me suis concentré sur la mise en évidence d'un nouveau rôle de la voie Toll de la drosophile dans la défense de l'hôte contre *A. fumigatus*.

J'ai d'abord établi que la souche d' *A. fumigatus* que j'utilise n'infecte les drosophiles que dans un modèle d'injection et non par ingestion ou contact avec les conidies, au rebours d'une étude précédente¹⁷. J'ai ensuite établi des courbes de survie du mutant *MyD88* (un gène essentiel de cette voie agissant en aval du récepteur Toll) en fonction de différentes doses injectées et montré qu'une dose croissante de conidies injectées entraîne une mort accélérée du mutant. J'ai donc travaillé généralement avec une dose de 250 conidies injectées par mouche. De manière inattendue, cette dose induit la voie Toll que très faiblement ainsi qu'en atteste l'induction de la *Drosomycine*. J'ai confirmé des résultats surprenants rapportés par une étude précédente, à savoir que le titre d' *A. fumigatus* n'augmente pas dans les mouches où la voie Toll est inactivée bien que ses effecteurs ne soient que peu induits¹⁷. J'ai aussi confirmé ces résultats par une approche histologique en observant directement une souche d' *A. fumigatus* exprimant une protéine fluorescente. Le champignon reste confiné au site d'injection, essentiellement sous formes d'hyphes. Il commence à émerger du thorax, site d'injection, quelques heures après le décès des mouches mutantes *MyD88*. Il s'agit donc plutôt d'un champignon nécrotrophe. Il semble donc qu'au rebours des autres infections caractérisées jusqu'à présent chez la drosophile, un nombre limité de conidies suffit à tuer les mouches mutantes pour la voie Toll. **La voie Toll ne contrôle pas la prolifération de ce champignon.** J'ai identifié la mélanisation comme étant une des voies essentielles du contrôle de la prolifération. Lorsque la mélanisation est bloquée, le champignon prolifère de manière significative dans les drosophiles, ce que j'ai pu déterminer en mesurant le titre et en observant la souche fluorescente *in vivo*. De manière frappante, il émerge de toutes les parties du corps 24 heures après la mort de leur hôte, de la tête à l'abdomen. Cependant, un mutant de mélanisation succombe nettement moins rapidement à l'infection, une dizaine de jours, qu'un mutant de la voie Toll, lequel succombe en deux-trois jours.

J'en conclus que l'essentiel n'est donc pas de contrôler la prolifération du champignon mais qu'une autre défense impliquant la voie Toll joue un rôle fondamental. Nous avons raisonné qu'il était vraisemblable que les mouches succombent non à l'invasion de leurs tissus par le champignon, mais suite à l'émission de toxines. Effectivement, un mutant d' *A. fumigatus* incapable de métabolisme secondaire, et donc de produire des toxines, perd sa virulence. Je n'ai pas pu confirmer une étude précédente et ai trouvé qu'un mutant incapable de produire la gliotoxine est aussi virulent dans nos conditions. L'injection de gliotoxine purifiée tue au même rythme les mouches sauvages et les mutants *MyD88*. L'injection de fumagilline ou d'acide hévolique a abouti à des résultats similaires. Cependant, d'autres toxines fongiques purifiées tuent les mutants *MyD88* plus rapidement que les mouches sauvages qui survivent en beaucoup plus grand nombre à leur injection; il s'agit des fumitromorgines, de dérivés de l'ergot du seigle, du verruculogène et de la restrictocine. ***Mes travaux nous amènent donc à conclure que la fonction essentielle de la voie Toll dans la défense de l'hôte n'est pas la résistance mais la résilience à l'infection,*** ce qui ouvre un nouveau domaine de recherche, étant donné l'importance des toxines fongiques vis-à-vis de la santé humaine, en particulier en ce qui concerne la contamination de la nourriture¹⁸. Il sera particulièrement intéressant de déterminer si ces résultats peuvent être étendus à l'homme.

J'ai mis au point un crible à grande échelle de lignées mutantes de drosophiles en testant leur survie à l'injection d'une dose de 250 conidies d' *A. fumigatus* injectée par mouches. Pour cela, nous utilisons une collection de lignées de drosophiles transgéniques, chaque lignée portant un microARN ciblant un gène spécifique du génome¹⁹. Afin d'éviter une létalité précoce liée à une éventuelle fonction du gène ciblé au cours du développement, les transgènes ne sont exprimés, ubiquitairement, qu'au stade adulte. Ce crible a nécessité la mise en place d'une infrastructure importante avec plusieurs techniciens s'occupant de l'entretien des souches mutantes et en charge d'effectuer les croisements. Comme il s'agit d'une organisation lourde, nous avons décidé d'optimiser ce crible en testant en parallèle la survie à *Metarhizium anisopliae* et à l'injection de trois virus, en collaboration avec une autre équipe. J'ai

pour ma part injecté lors d'un pré-crible près de 600 lignées et procédé au retest d'une trentaine de lignées positives. Lors du crible principal, j'ai testé 3967 lignées, la grande majorité des retests ayant été réalisés par Mme Chuqin Huang qui avait pris ma suite. A présent, plus de 6471 lignées ont été criblées, lesquelles ciblent collectivement environ la moitié des gènes codant des protéines du génome de la drosophile; 1163 lignées ont été retestées, et 249 lignées confirmées au cours de ces retests. Un des premiers mutants identifié affecte la Cycline K, un facteur qui, complexé avec Cdk12, est impliqué dans l'élongation de la transcription d'un ensemble de gènes régulés par la voie Nrf2^{20,21}. Ce dernier facteur de transcription est le chef d'orchestre de la réponse anti-oxydante de l'organisme, ainsi qu'un facteur requis pour la détoxification de certains composés xénobiotiques²². Alors que les mutants *Nrf2/CncC* sont effectivement plus sensibles à l'infection par *A. fumigatus*, comme les mutants *cdk12*, ils ne présentent toutefois pas une susceptibilité particulière à l'injection de toxines fongiques. Ainsi, nous serons amenés à tester l'importance des réactions oxydatives et de la détoxification de leurs effets néfastes sur l'organisme dans la défense de l'hôte contre *A. fumigatus*.

En conclusion, mon travail de thèse a permis une avancée significative dans notre compréhension des défenses de l'hôte contre *A. fumigatus* en soulignant l'importance de la résilience vis-à-vis des toxines fongiques. Le crible génétique à large échelle en cours devrait nous permettre de mieux comprendre les différents aspects de la défense de l'hôte contre ce champignon pathogène et contre les toxines fongiques.

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la figure de légendes

figure 1: a. *fumigatus* ne prolifèrent dans *myd88* mutants

a: courbe de dose - réponse, sur *myd88* s'en sert af faire l'injection, il n'y a pas de différence significative entre le pbst et 1 conidie par voler groupe ($p = 0.9230$), mais avoir de différence significative entre le pbst et 5 conidie par voler group ($p < 00001$).les données ont été analysées au moyen du test log - rank.

b: le champ clair chez le type sauvage en hyphes fluorescentes *gfp*, b « même poste à filtre, d141-*gfp* 50 de conidies par voler l'injection, 20h après l'infection (flèche).

c: d141-*gfp*50 hyphes fluorescentes dans *myd88* conidies par voler mouches, injection, 24 h après l'infection (flèche).

d: dans *myd88* hyphes fluorescentes mouches, d141-*gfp* 500 de conidies par voler l'injection, 42h après l'infection (flèche).

e: la charge fongique à faible dose, af 250 de conidies par voler l'injection, chez les groupes, 0h ont aucune différence significative à 24h ($p = 0.8139$) et 48 h ($p = 0.3703$); dans *myd88* groupes, 0h ont sensiblement 24h ($p < 00001$) mais pas à 48 heures ($p = 0.1033$).les données ont été analysées au moyen du test de mann - whitney.

f: la charge fongique haute dose, af 5000 de conidies par voler l'injection, il existe une différence importante entre le wt 0h et 48 h ($p < 00001$), mais aucune différence significative entre myd88 0h et 48 h ($p = 0.1009$).les données ont été analysées au moyen du test de mann - whitney.

g: sérum dans myd88 vole, af injection, $10 [7] = 50$ de conidies par voler, $5 \times 10 [7] = 250$ de conidies par voler, et $10 [8] = 500$ de conidies par voler, il y a une différence entre 0h et de sérum dans $10 [7]$ ($p < 00001$) et $5 \times 10 [7]$ ($p = 00002$), mais pas dans $10 [8]$ ($p = 0.7821$).les données ont été analysées au moyen du test de mann - whitney.

h: im1 niveau d'expression de vivre et tué a. fumigatus conidies injection, à différentes concentrations de $10 [7] = 50$ de conidies par voler, $10 [8] = 500$ de conidies par voler.il n'y a pas de différence significative entre les af $10 [8]$ et wt m. lutues ($p = 0.9314$), d'autres groupes ont sensiblement wt af $10 [8]$ et wt m. luteus groupes ($p < 00001$).les données ont été analysées au moyen du test de mann - whitney.

figure 2: hayan, et pas les autres défenses de l'hôte, limite la diffusion de a. fumigatus dans la mouche

un taux de survie: clé, af 500 de conidies par voler l'injection, il existe une différence importante entre les groupes et l'aide ($p < 00001$).les données ont été analysées au moyen du test log - rank.

b: la charge fongique, af 500 de conidies par voler l'injection. il y a une différence entre 0 h et 48 h / 96 h après groupe infecté dans les mouches ($p < 00001$), mais pas dans myd88 mouches ($p = 0.5574$). les données ont été analysées au moyen du test de mann - whitney.

c: eater1 et manger Δ eater survival, af 500 de conidies par voler l'injection, il n'y a pas de différence significative entre Δ eater les eater1 et manger des mouches et wt ($p = 0.9990$). les données ont été analysées au moyen du test log - rank.

d: perles de latex survies wt traitées, af 500 de conidies par voler l'injection, il n'y a pas de différence significative entre les perles de latex traités wt mouches et wt ($p = 0.9992$). les données ont été analysées au moyen du test log - rank.

e: phago hemoless samu rpr samu a caché la survie, af 500 de conidies par voler l'injection, il n'y a pas de différence significative entre phago hemoless mouches et wt ($p = 0.9984$). les données ont été analysées au moyen du test log - rank.

f: hayan survival, d141-gfp 500 de conidies par voler l'injection, bleu rouge courbes courbes sont myd88 wt des mouches, mouches, green courbes sont hayan mouches, il y a des différences significatives entre les groupes myd88 et wt infectés à hayan groupe infecté ($p < 00001$). les données ont été analysées au moyen du test log - rank.

g: sérum hayan, d141-gfp 500 de conidies par voler l'injection, il existe d'importantes différences entre 0 h après l'infection et le sérum de hayan mouches ($p < 00001$), mais aucune différence significative dans myd88 mouches ($p = 0.7030$). les données ont été analysées au moyen du test de mann - whitney.

h: papa survival, af 250 de conidies par vole d'injection, bleu rouge courbes courbes sont myd88 wt des mouches, mouches, courbes de vert papa vole, il existe d'importantes différences entre papa et wt mouches dans les af groupes infectés ($p < 00001$).les données ont été analysées au moyen du test log - rank.

- je: balayage d'hyphes (arrow) sur la surface du corps myd88 de mouches, 48 heures après la mort.

j: balayage em des hyphes (arrow) sur la surface du corps hayan de mouches, 48 heures après la mort.

figure 3: myd88 est sensible à certaines toxines a. fumigatus

un: Δ ppta survie, les conidies d'af et Δ ppta était injecté dans myd88 500 mouches, les conidies par voler, il existe une différence importante entre l'af et Δ ppta groupes ($p < 00001$).les données ont été analysées au moyen du test log - rank.

b: uvitex coloration négative des hyphes (flèche).

c: charge fongique du Δ ppta, 500 de conidies par voler l'injection, voici les différences importantes entre 0 et 48 heures après l'injection de Δ ppta mutant a. fumigatus en poids ($p < 00001$) et myd88 mouches ($p = 00001$).les données ont été analysées au moyen du test de mann - whitney.

d: dose - response de la restrictocine injection, bleu rouge courbes courbes sont myd88 poids mouches, mouches, il y a une différence entre myd88 et wt vole dans toutes les concentrations (1, 5, 10 mg / ml) la restrictocine injection groupes ($p < 00001$).les données ont été analysées au moyen du test log - rank.

e: la dose - réponse de le verrucogène injection

Figure 1

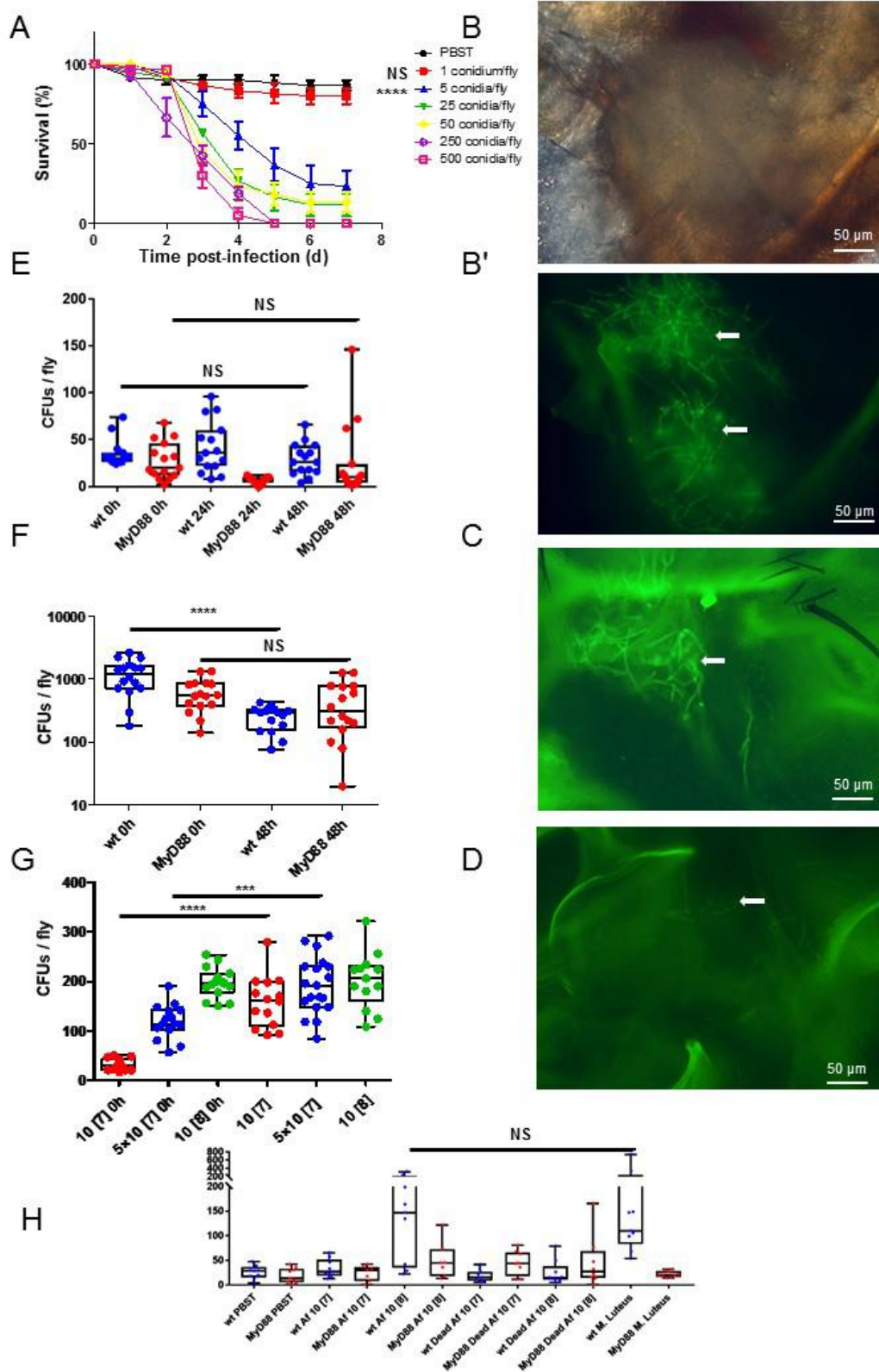


Figure 2

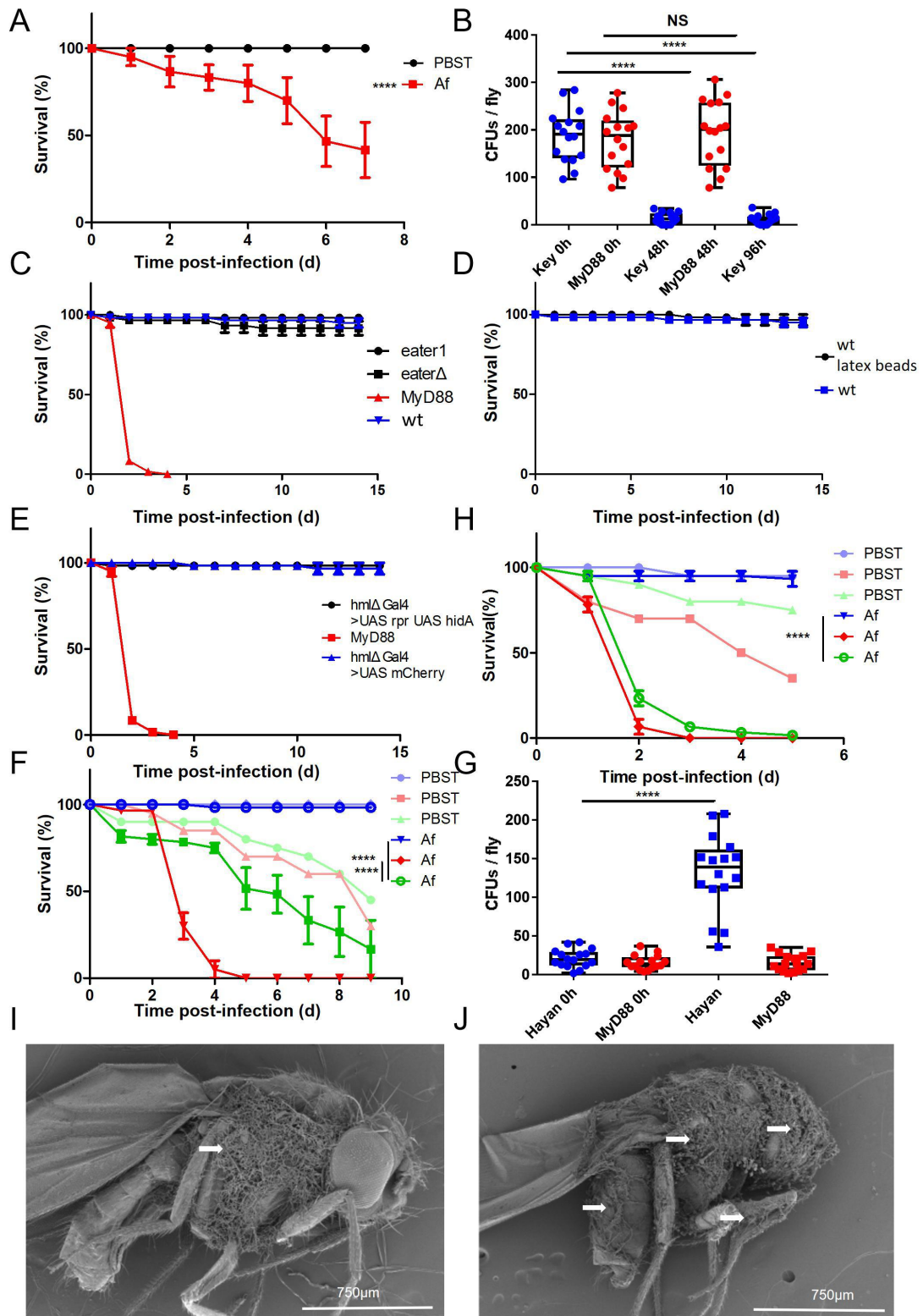


Figure 3

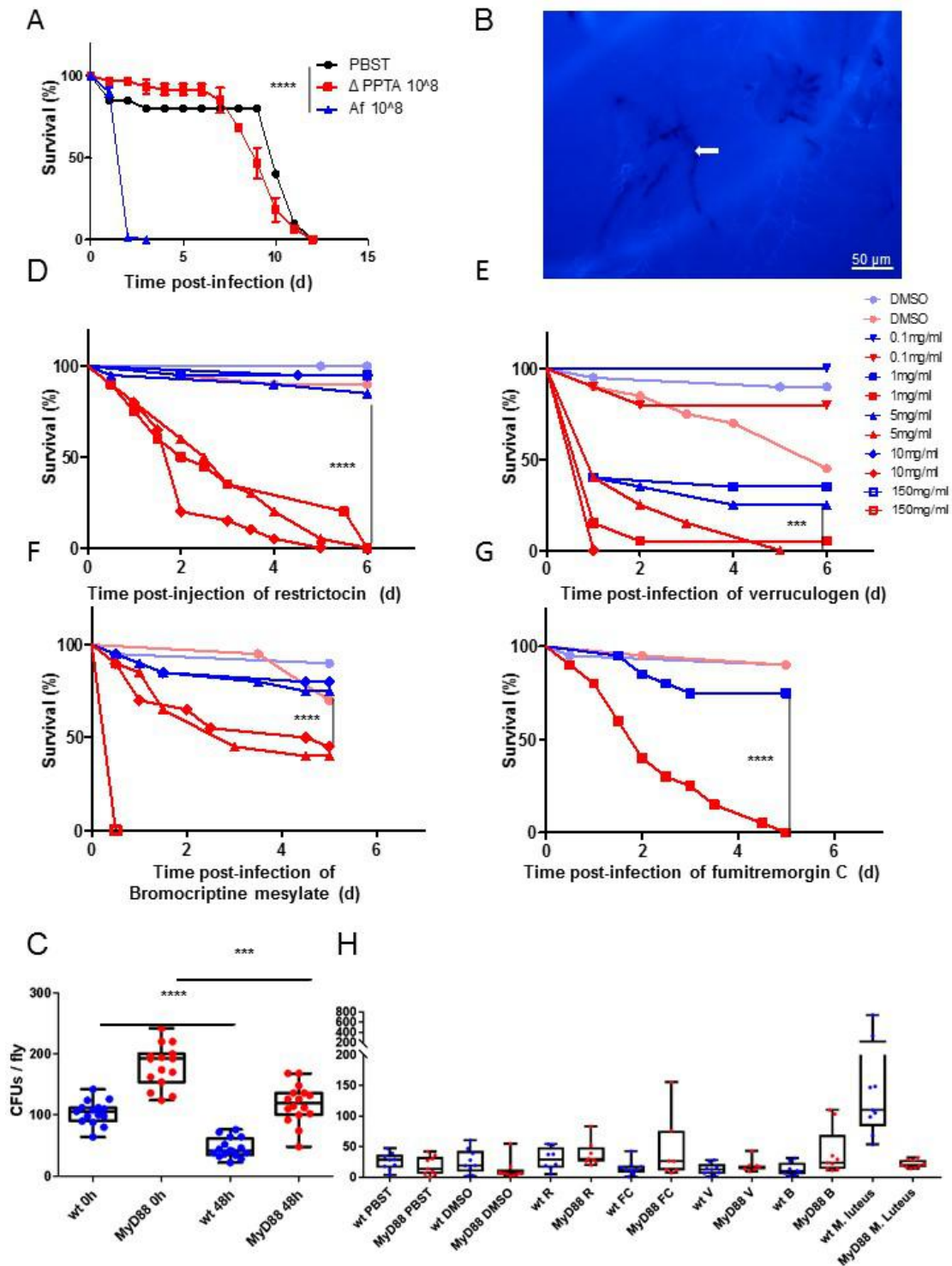


fig s1: wt vole la dose - réponse et l'essai de différentes souches de *a. fumigatus* myd88.

a: wt vole la dose - réponse, l'utilisation af faire l'injection, il n'y a pas de différence significative entre les différentes lignes de voler le ($p = 0.9880$).les données ont été analysées au moyen du test log - rank.

b: survie de différentes souches de *a. fumigatus* myd88, il n'y a pas de différence significative entre les différentes souches de *a. fumigatus* ($p = 0.9972$).les données ont été analysées au moyen du test log - rank.

fig. s2: fluorescent (flèche d'hyphes dans hayan vs. myd88).

fig.s3: la gliotoxine n'est pas nécessaire ni suffisante pour le myd88 tuer des mouches.

un: la survie de la gliotoxine mutant Δ glip et son contexte cea17 Δ akubku80.les courbes sont le bleu myd88 mouches, mouches courbes de rouge, myd88, il n'y a pas de différence significative entre les différentes concentrations de Δ glip et cea17 Δ akubku80 ($p = 0.9990$).les données ont été analysées au moyen du test log - rank.

b: survie de la gliotoxine directement l'injection.les courbes sont le bleu myd88 mouches, mouches courbes de rouge, myd88, il n'y a pas de différence significative entre les concentrations différentes de la gliotoxine injection ($p = 0.9890$).les données ont été analysées au moyen du test log - rank.

tableau s4: helvolic injection d'acide et de la fumagilline

un: la survie de l'acide helvolic directement l'injection.les courbes sont le bleu myd88 mouches, mouches courbes de rouge, myd88, il n'y a pas de différence significative entre les différentes concentrations d'acide helvolic injection ($p = 0.9763$).les données ont été analysées au moyen du test log - rank.

b: survie de la fumagilline directement l'injection.les courbes sont le bleu myd88 mouches, mouches courbes de rouge, myd88, il n'y a pas de différence significative entre les concentrations différentes de la fumagilline injection ($p = 0.9991$).les données ont été analysées au moyen du test log - rank.

fig. s5: la température est un facteur qui impliquent dans les deux toxines a. fumigatus infection et l'injection.

un: la survie dans des températures différentes, af 500 de conidies par voler l'injection.les courbes sont le bleu myd88 mouches, mouches courbes de rouge.

b: survie de l'injection de toxine à différentes températures sur myd88 des mouches.

fig. s6: charge bactérienne dans l'hémolymph de mouches ".

a: charge bactérienne dans l'hémolymph des mouches "après 48 heures après l'injection, 5 des mouches pour chaque endroit.

b: charge bactérienne dans l'hémolymph des mouches "après 48 heures après l'injection, seul vol test.

Fig. s7: des antibiotiques et des mouches

un: la survie de la restrictocine injection au traitement normal (ab) et axéniques des mouches.

b: survie de le verruculogène injection normal, antibiotiques traités (ab) et axéniques de mouches.

c: survie de la bromocriptine mésylate (un dérivé d'alcaloïdes de l'ergot de seigle) injection au traitement normal (ab) et axéniques des mouches.

d: survie de fumitremorgin c injection au traitement normal (ab) et axéniques des mouches.

Figure S1

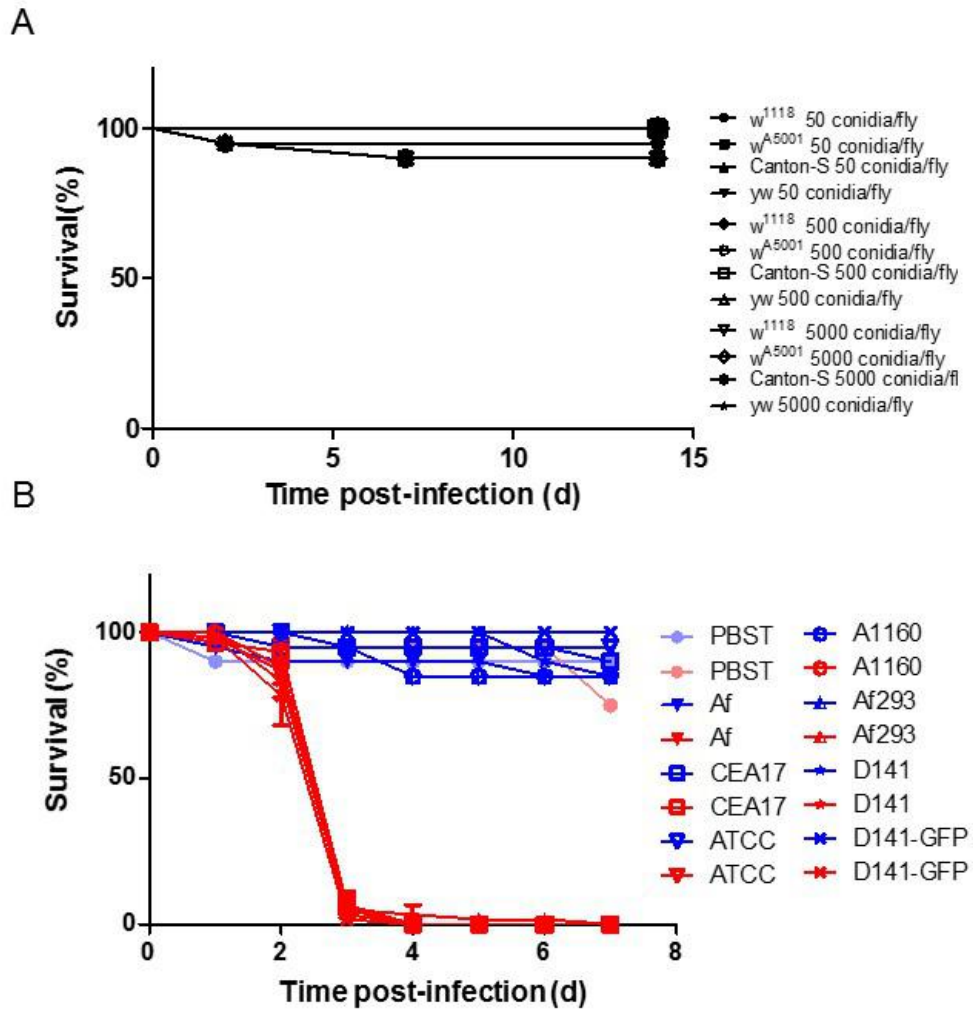


Figure S2

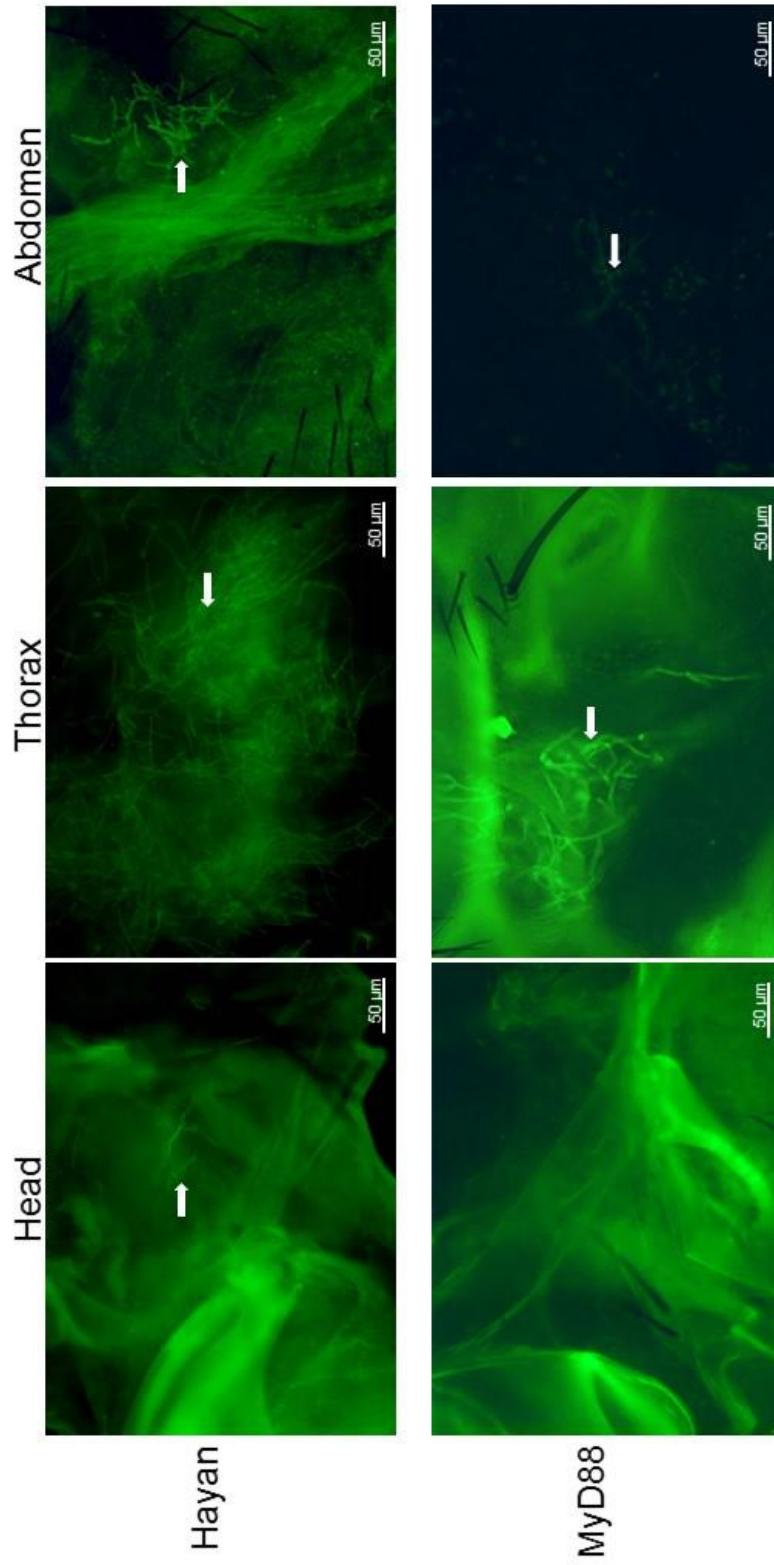
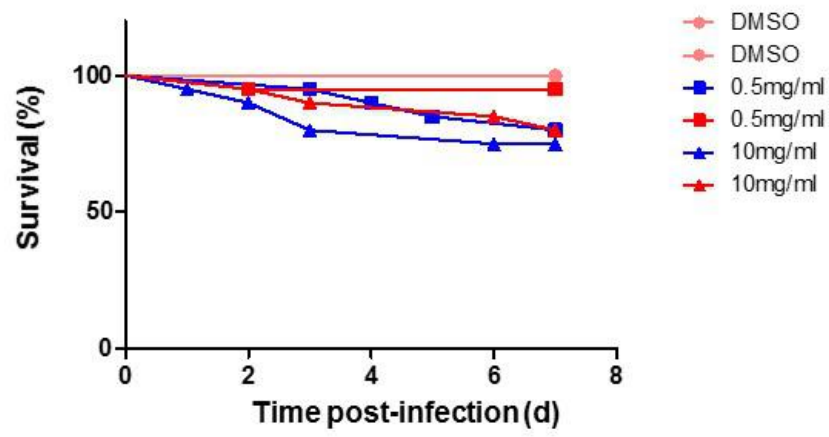


Figure S4

A



B

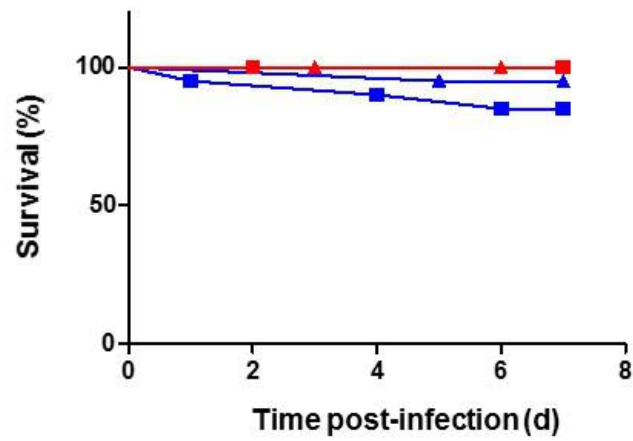
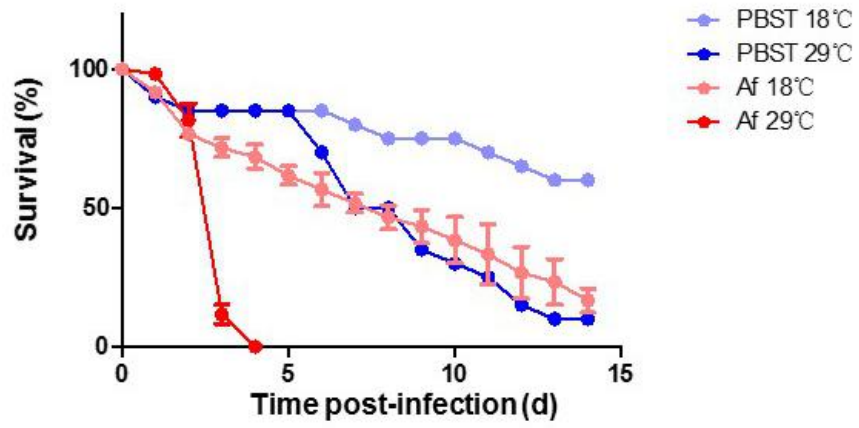


Figure S5

A



B

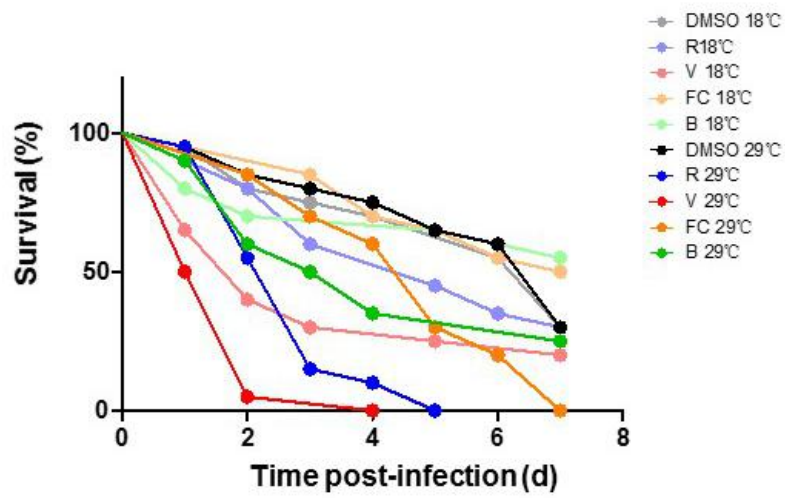


Figure S7

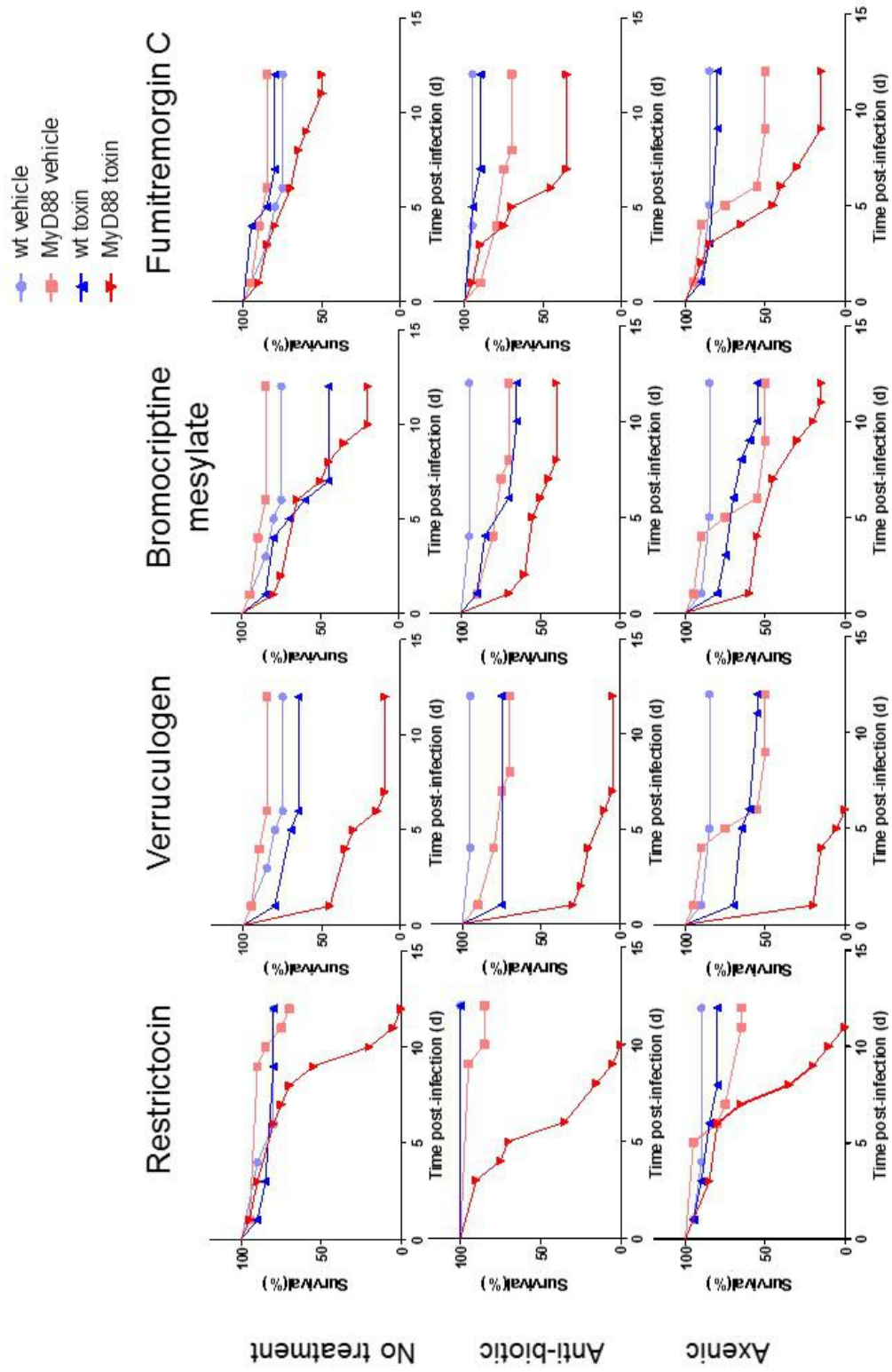


Table 1

	head	thorax	abdomen
<i>MyD88</i>	0% (0/30)	50% (50/100)	1% (1/100)
<i>Hayan</i>	42.5% (17/40)	100% (40/40)	95% (38/40)

Tableau 1: quantification de la présence de mycélium après infection.

Résumé

Le but de ce travail a été de mieux comprendre les défenses mises en œuvre par l'hôte infecté par le champignon opportuniste humain *Aspergillus fumigatus* (*Af*).

1) Un modèle d'infection a été redéveloppé chez l'organisme modèle *Drosophila melanogaster*. Seules les mouches mutantes pour le gène *MyD88* de la voie immunitaire Toll succombent à l'injection d'une poignée de conidies, sans toutefois qu'*Af* dissémine dans l'hôte. **Ce travail a révélé que ce n'est pas la réponse immunitaire qui joue un rôle prépondérant dans la défense de l'hôte, mais sa capacité de résilience à l'exposition à des mycotoxines sécrétées par *Af*.**

2) Un crible génétique d'envergure a été établi pour identifier des lignées transgéniques mutantes ARNi sensibles à l'infection par *Af*. 6.471 lignées ont été criblées et 241 gènes-candidats identifiés, dont peu fonctionnent dans la réponse immunitaire. Ainsi, ce travail a contribué à identifier de nombreux gènes impliqués dans la résilience de l'hôte à *Af* et ses mycotoxines.

Mots-clés : *Aspergillus fumigatus*, *Drosophila melanogaster*, voie Toll, résilience à l'infection, mycotoxine

Résumé en anglais

The overarching goal of this work is to better understand host defenses against the human opportunistic fungus *Aspergillus fumigatus* (*Af*).

1) An infection model has been reestablished in the genetic model organism *Drosophila melanogaster*. Only flies mutant for the immune response Toll pathway gene *MyD88* succumb to the injection of a handful of conidia even though *Af* is unable to disseminate throughout its host. **This work revealed that it is not the immune response that plays a cardinal role in host defense but its resilience capacity to the exposure to some mycotoxins secreted by *Af*.**

2) A large-scale genetic screen has been implemented to identify transgenic RNAi mutant lines susceptible to *Af* infection in survival experiments. 6,471 lines have been screened and 241 candidate genes identified, few of which are known to act in the immune response. Thus, this work has contributed to identifying numerous genes involved in host resilience to *Af* and to some of its mycotoxins.

Key words: *Aspergillus fumigatus*, *Drosophila melanogaster*, Toll pathway, Resilience to infection, Mycotoxin