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GENETICS OF IMMUNITY

A Host-Pathogen Interaction Screen Identifies *ada2* as a Mediator of *Candida glabrata* Defenses Against Reactive Oxygen Species

Ilias Kounatidis,* Lauren Ames,† Rupal Mistry,* Hsueh-lui Ho,† Ken Haynes,† and Petros Ligoxygakis*,1
*Cell Biology, Development and Genetics Laboratory, Department of Biochemistry, University of Oxford, OX1 3QU UK and †Exeter Biosciences, College of Life and Environmental Sciences, University of Exeter, EX4 4QD, UK
ORCID ID: 0000-0001-9877-0457 (I.K.)

ABSTRACT *Candida glabrata* (*C. glabrata*) forms part of the normal human gut microbiota but can cause life-threatening invasive infections in immune-compromised individuals. *C. glabrata* displays high resistance to common azole antifungals, which necessitates new treatments. In this investigation, we identified five *C. glabrata* deletion mutants (Δ*ada2*, Δ*bas1*, Δhir3, Δ*ino2* and Δ*met31*) from a library of 196 transcription factor mutants that were unable to grow and activate an immune response in *Drosophila* larvae. This highlighted the importance of these transcription factors in *C. glabrata* infectivity. Further *ex vivo* investigation into these mutants revealed the requirement of *C. glabrata ADA2* for oxidative stress tolerance. We confirmed this observation *in vivo* whereby growth of the *C. glabrata Δada2* strain was permitted only in flies with suppressed production of reactive oxygen species (ROS). Conversely, overexpression of *ADA2* promoted *C. glabrata* replication in infected wild type larvae resulting in larval killing. We propose that *ADA2* orchestrates the response of *C. glabrata* against ROS-mediated immune defenses during infection. With the need to find alternative antifungal treatment for *C. glabrata* infections, genes required for survival in the host environment, such as *ADA2*, provide promising potential targets.

KEYWORDS
Candida
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Candida glabrata is a small, asexual, haploid yeast and is the second most frequent cause of candidiasis after *Candida albicans*, accounting for ~15–25% of clinical cases (Perlroth *et al.* 2007; Méan *et al.*, 2008; Pfaller *et al.*, 2010). Despite its normally asymptomatic presence in the human gut microbiota, it can cause severe invasive infections in immune-compromised individuals and hospitalized patients (Méan *et al.*, 2008). Such infections are associated with high mortality rates and prolonged hospital stays, consequently increasing healthcare costs. A number of risk factors including the use of central venous catheter devices and treatment with antibiotics have been associated with the development of candidiasis (Perlroth *et al.* 2007).

In agreement with its phylogenetic position closer to the baker's yeast *Saccharomyces cerevisiae* than to *C. albicans* (Massey *et al*; 2003; Dujon *et al.*, 2004; Roetzer *et al.* 2011), *C. glabrata* lacks many attributes believed to be key mediators of fungal pathogenicity in other *Candida* species such as the secretion of hydrolytic enzymes and the ability to form hyphae (Butler *et al.*, 2009; reviewed in Gow *et al.*, 2011). A primary virulence attribute of *C. albicans*, the leading cause of Candidiasis, is its ability to switch from a yeast to filamentous form upon certain environmental cues, which enables *C. albicans* to actively penetrate host cells. These hyphae extend filaments into the host cells, releasing hydrolytic proteases and lipases, which lead to the eventual disruption of host cellular function (Ghannoum 2000; Albrecht *et* al 2006; Wächtler *et al.*, 2012). *C. glabrata* however, is haploid and can only grow in the yeast form. Therefore, with the absence of hyphal formation, the mode of invasion of *C. glabrata* is probably different from that of *C. albicans*.

Nonetheless, *C. glabrata* is still pathogenic to humans and therefore must rely on other distinct strategies to invade and persist in infected individuals. Although the exact mode of entry is unclear, the ability of *C. glabrata* to invade host tissue was demonstrated in a chicken embryo

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[1]Corresponding author: Cell Biology, Development and Genetics Laboratory, Department of Biochemistry, University of Oxford, South Parks Rd, OX1 3QU UK. E-mail: petros.ligoxygakis@bioch.ox.ac.uk

model of infection where *C. glabrata* cells were found to cross the chlorio-allantoic membrane (CAM) (Jacobsen *et al.*, 2011). In this context, a way in which *C. glabrata* may gain entry to host tissues is through pseudo-hyphal growth, which has been reported *in vitro* (Csank and Haynes 2000). *In vivo*, *C. glabrata* persists through endocytosis with hardly any host cell damage (Li *et al.*, 2007). Because of this low host cell damage, the cytokine profile of *C. glabrata*-infected epithelia differs significantly from that of *C. albicans*-infected cells (Li *et al.*, 2007). As a result, this leads to a strong neutrophil infiltration typical of *C. albicans* infection whereas infection with *C. glabrata* is associated with mononuclear cells (Westwater *et al.*, 2007; Jacobsen *et al.*, 2010). Once within the host, survival and the establishment of infection depends on the ability of *C. glabrata* to mount efficient responses to a changing, stressful environment; to acquire often limited nutrients; to evade the immune response.

The intrinsic resistance of *C. glabrata* to oxidative stress is of particular note (Cuéllar-Cruz *et al.*, 2008; Kaloriti *et al.*, 2012). The oxidative burst elicited by immune cells is a first line of defense against invading microorganisms (reviewed in Fang 2004). In turn, the ability of the pathogen to detoxify such reactive oxygen species (ROS), via the expression of detoxifying enzymes (catalase and superoxide dismutase) and production of antioxidants glutathione and thioredoxin, is important for surviving immune attack (reviewed in Brown *et al.*, 2009). For example deletion of *SKN7*, a transcription factor mediating the oxidative stress response, attenuates *C. glabrata* virulence in a murine model of disseminated candidiasis (Saijo *et al.*, 2010). This suggests additional mechanisms that contribute to increased oxidative stress resistance in *C. glabrata* beyond what we know from *S. cerevisiae*. Therefore, knowing the gene networks orchestrating *C. glabrata* responses to the host environment would be highly beneficial in understanding *C. glabrata* infection biology. Once *C. glabrata* has expanded to internal organs, its high resistance to common azole antifungals makes it hard to treat (Pfaller *et al.*, 2010). High cost antifungals including echinocandins (*e.g.*, caspofungin), anidulafungin and micafungin can also induce resistance (see Perlin 2007; Perlin 2014; Singh-Babak *et al.* 2012).

In light of the increased incidence of drug resistant *C. glabrata* infections, successful alternative treatment of *C. glabrata* perquisites further investigation of its pathogenicity. To this direction current mammalian models do not provide the most ideal approach to identify virulence factors because of their high cost, labor-intensive procedure, low statistical resolution and ethical concerns. Nonetheless, virulence factors in *C. glabrata* have been identified in these systems, including adhesins and cell-bound proteases (Cormack *et al.*, 1999; Kaur *et al.*, 2007; Tsoni *et al.*, 2009; Seider *et al.*, 2011). However, as large genomic libraries with deletion mutants become available in clinically relevant *Candida* pathogens, invertebrates provide an alternative approach for individually investigating virulence factors and host immune responses. *Drosophila* is a small, inexpensive to rear and easy to manipulate model organism with a variety of sophisticated genetic tools and a fully annotated genome. Additionally, its interaction with *Candida* has been studied extensively, making it an excellent system for screening antifungals, investigating pathogenicity and identifying novel virulent factors in both systemic (injection) as well as gastrointestinal (feeding) infection (Brunke *et al.*, 2015; Glittenberg *et al.*, 2011a; Glittenberg *et al.*, 2011b; Alarco *et al.*, 2004; Chamilos *et al.*, 2006).

The *Drosophila* Toll pathway is activated upon encountering a fungal infection (Lemaitre *et al.*, 1996). Glucan-binding protein (GNBP3) binds to fungal β-1,3-glucan that activates a proteolytic cascade that results in cleavage of Spätzle (Gottar *et al.*, 2006). Additionally, the protease Persephone (PSH) is also able to detect the activity of microbial virulence factors and can trigger the Toll pathway (Gottar *et al.*, 2006). The signal is transmitted in the nucleus through the transcription factor, Dorsal-related immunity factor (DIF), which regulates targets genes including induction of the antimicrobial peptide (AMP) gene *drosomycin* (*drs*) (Rutschmann *et al.*, 2000). An additional pathway in epithelial immunity is the Imd pathway (for immune deficiency). There, a transmembrane or intracellular peptidoglycan recognition protein (PGRP-LC and PGRP-LE respectively) forms a receptor-adaptor complex with IMD itself (a RIP1 homolog), which associates with FADD (the Fas-associated death domain protein) then recruiting the caspase-8 homolog DREDD (reviewed in Kounatidis and Ligoxygakis 2012). This receptor proximal complex acts as a platform for the recruitment and activation of the TAK1 kinase, which is presumed to phosphorylate the IκB-Kinase (IKK). In turn, IKK (IKKα and IKKγ-otherwise known as Kenny) phosphorylates the N-terminal domain of the NF-κB homolog Relish, while DREDD cleaves the C-terminal (Paquette *et al.*, 2010). N-terminal Rel is then free to move to the nucleus and regulate transcriptional targets including induction of antimicrobial peptide (AMP) genes (Stöven *et al.* 2000). In addition, Reactive Oxygen Species (ROS) are an important defense in controlling microbial invasion in the gut, regulated by the *Drosophila* dual oxidase gene (dDuox) (Ha *et al.*, 2005).

Previous work from our laboratory involved *Drosophila* as a model organism to investigate pathogenicity of *C. albicans* following gastrointestinal (GI) infection (Glittenberg *et al.*, 2011a; 2011b). We determined that GI infection of wild type larvae with wild type *C. albicans* resulted in 83% of larvae reaching adulthood whereas 70% of larvae deficient for NF-κB-driven pathways developed into adults. In particular, the gut commensal bacteria community was beneficial as only 58% of germ-free larvae survived to adults while even less immune-compromised germ-free larvae (22%) developed into adults following infection with *C. albicans* (Glittenberg *et al.*, 2011b). Despite *C. albicans* being restricted to the gut, it still caused systemic infection as detected by *drs* expression from the fat body.

Using a similar feeding protocol, we established a GI infection model for *C. glabrata* in *Drosophila* larvae. We screened (one at a time) a library of deletion mutants including 196 *C. glabrata* transcription factors (Schwarzmüller *et al.*, 2014) and we identified five that did not grow or activate systemic immunity in larvae. From these five fungal mutants, *C. glabrata* deficient for ADA2 was highly sensitive to very low concentrations of H_2O_2 (a hallmark of oxidative stress) *ex vivo*. Inside the host, the *ada2* mutant was only able to grow in ROS-suppressed *Drosophila* while wild type *C. glabrata* with an additional copy of *ada2* was able to grow and kill wild type larvae. Our results suggest that ADA2 mediates *C. glabrata* defenses against ROS and is therefore a potential target to diminish *C. glabrata* growth during GI infection.

MATERIALS AND METHODS

Drosophila stocks and genetics

The following stocks were used: w^{1118} (BL #6326), *Drs-GFP* (Ferrandon *et al.*, 1998), *dif-key* (Rutschmann *et al.*, 2002), OregonR. Mutant strains were isogenised by backcrossing 10 times in w^{1118} background, thus refer in the manuscript as w^{1118}; *drs-GFP* and w^{1118}; *dif-key*.

Infection experiments

We followed a modified gastrointestinal infection model (Glittenberg *et al.*, 2011b) based on a previously established bacterial oral infection protocol (Basset *et al.*, 2000). Accordingly, every *C. glabrata* mutant strain was tested one-by-one. Prior to infection, 10mL of yeast extract peptone dextrose (YPD) (Y1375, Sigma-Aldrich, USA) was inoculated

with a single colony of yeast and incubated on a rotating shaker at 30° at 120 rpm for 16 hr. The inoculum was pelleted by centrifuging at 4° at 4000 rpm for 4 min and the supernatant was discarded. The pellet was washed in 10mL of 1X PBS and centrifuged again at 4° at 4000 rpm for 4 min and the supernatant was discarded. A single banana was homogenized and 450μL was added to 2mL eppendorf tubes. To this, 250μL of pathogen (OD = 200) was also added. As a control, banana without pathogen was used. Five-day (third instar) larvae were collected by washing from the fly food and caught in a sieve. They were starved for one hour before infection. ~100 larvae were added to each eppendorf tube, plugged with breathable foam with space for fermentation and allowed to feed for 30 min. The mixture then was transferred to a standard fly medium and incubated at 30°. Lethality of larvae following infection was determined by counting surviving pupae. 48 hr after infection.

TFKOs mutants screen

The transcription factor knockout (TFKO) library screen was conducted following the above oral infection protocol in w^{1118}; *drs-GFP* 5-day old larvae. Two days after infection, pupae were visualized under UV light to confirm that they were unable to activate immunity. Further to this, *drs-GFP* were infected with the complement strain of each hit to re-confirm that when the knocked-out gene was re-inserted, immunity would be activated.

Yeast counts from larvae by counting CFUs

Following infection, larvae were washed in 100% ethanol, rinsed in sterile water and transferred to normal fly food. Larvae were homogenized (at indicated time points) in 200 μL YPD media (Y1375, Sigma-Aldrich, USA). Serial dilutions were made and 50 μL, plated on YPD plates (Y1500, Sigma-Aldrich, USA). Plates were incubated at 30o and colonies were measured accordingly.

Microscopy

Larvae were visualized on a GFP stereo dissecting microscope (Leica MZFIII, UK), and images captured using KyLink software (v2.0, Japan).

Gene expression analysis

Drosomycin expression was determined from five independent biological samples consisting of 5 infected third instar larvae following infection. Samples were compared to banana-fed flies of the corresponding time point. Larvae were collected at the desired time points and washed in 100% ethanol and sterile water. RNA was extracted using Purification Plus Kit (48400, Norgen – Biotek, Canada) and cDNA was prepared from 0.5 μg total RNA using Maxima First Strand cDNA Syntesis Kit (K1672, Thermo Scientific, UK). Triplicate cDNA samples were amplified with the SensiFASAT SYBR No-ROX Kit (BIO-98020, Bioline, UK) in a Corbet Rotor-Gene 6000 QPCR machine (Qiagen, UK) according to the manufacturer's protocols. Expression values were calculated using the DDCt method and normalized to rp49 expression levels (Schmittgen and Livak 2008).

Primers Used: rp49(forw): AAGAAGCGCACCAAGCACTTCATC, rp49(rev): TCTGTTGTCGATACCCTTGGGCTT, drosomycin(forw): AGTACTTGTTCGCCCTCTTCGCTG, drosomycin(rev): CCTTGTATCTTCCGGACAGGCAGT.

Measuring production of reactive oxygen species (ROS)

The amount of ROS was measured by quantifying the amount of hydrogen peroxide produced per larvae immediately after oral infection. The samples included three independent biological samples of 10 third instar larvae from non-infected, banana-fed and infected with C. glabrata samples. The amount of hydrogen peroxide produced from the sample was determined using Amplex red hydrogen peroxide/peroxidase assay kit (A22188, Invitrogen, USA) following manufacturer's instructions.

C. glabrata culture conditions

C. glabrata strains were cultured in YPD (1% yeast extract, 2% bacteriological peptone, 2% glucose) at 30°. A final concentration of 200 μg/ml nourseothricin (Werner BioAgents) was added for selection of C. glabrata deletion mutants. Complementation and overexpression strains were cultured in SC broth (0.69% yeast nitrogen base without amino acids, 2% glucose) supplemented with CSM single drop out (-His) mixture (Formedium). YPD and SC plates contained 2% agar.

C. glabrata deletion mutant construction

Construction of C. glabrata transcription factor deletion mutants used in this study was previously described (Schwarzmüller *et al.*, 2014).

C. glabrata complementation and overexpression strain construction

C. glabrata ORFs previously cloned into the pDONR221 entry vector using GATEWAY cloning technology (Thorne *et al.*, 2011) were shuttled into a pAG423GPD-ccdB destination vector (AddGene) using LR clonase. Destination vectors carrying the C. glabrata ORF were transformed by electroporation into the corresponding C. glabrata deletion mutant for complementation or into the parental C. glabrata Δhis3 strain for overexpression. Empty pAG423GPD-ccdB vector was transformed into C. glabrata as a control. Correct transformants were selected for growth on SC –his media. Three independent transformants of each strain were collected.

Phenotypic screening

Overnight cultures of C. glabrata strains were normalized to OD600 0.1 in sterile water. The normalized suspensions were aliquoted into a 96-well plate and diluted ten-fold across six wells to create serial dilutions. Using a multichannel pipette, 5 μl aliquots of the serial dilutions were spotted onto SC agar plates supplemented with stress-inducing chemical agents (Table S2). Plates were incubated at 30°, unless otherwise stated, and imaged daily. Upon visual inspection, phenotypes for each mutant were scored relative to the parental strain into six categories: mild sensitivity (MS), sensitive (S), severe sensitivity (SS), no growth (NG), improved growth (IG) or no phenotype. Three biological replicates were performed for all phenotypic screens.

Growth analysis

Overnight cultures of C. glabrata were normalized to OD600 0.1 in fresh YPD and distributed into a flat-bottomed 96-well plate in 100 μl aliquots. Optical density readings were taken every 10 min in a VersaMax Absorbance Microplate Reader set to 30° with shaking between reads. Growth rate was measured by calculating the doubling time for each mutant during exponential phase (OD600 0.4 – 0.8).

Data availability

Drosophila and *Candida* strains are available upon request. All data pertaining to this study can be found in the figures and supplemental material. A list of the *C. glabrata* mutants used for the primary screen can be found in table S3 of Schwarzmüller *et al.* 2014.

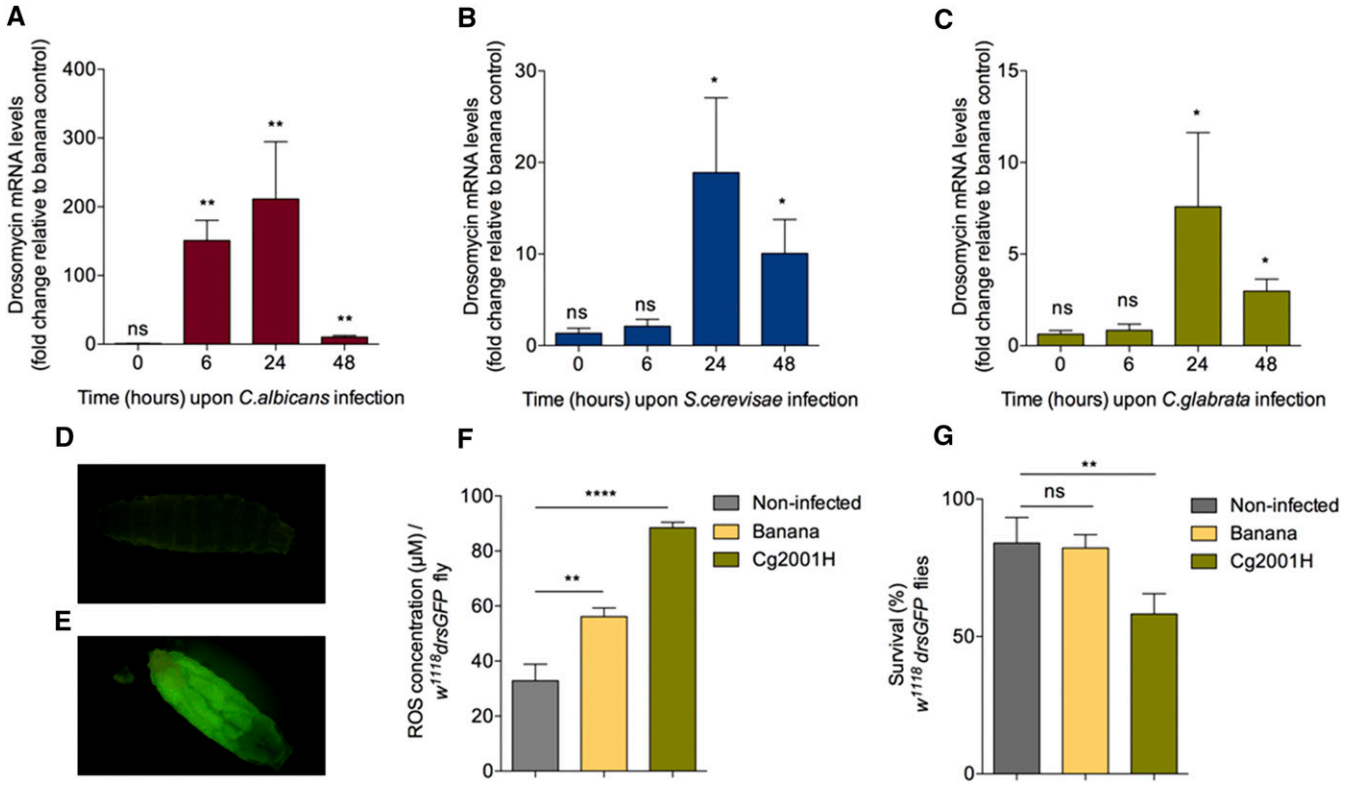

Figure 1 The host's response to gastrointestinal fungal infection. Quantification of *drs* mRNA levels following oral infection of w^{1118} with wild type yeast strains at selected time points (0, 6, 24 and 48 hr). Infection with (A) *C. albicans*, (B) *S. cerevisiae* and (C) *C. albicans*. *Drosomycin* levels at each time point are relative to the banana control of the corresponding time point. Error bars represent the standard deviation of 5 separate experiments; ns $P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$ indicate significant values when compared to banana control of the corresponding time point. (D) Non-infected w^{1118};*drsGFP* white pre-pupae visualized under a fluorescent stereoscope. (E) w^{1118};*drsGFP* white pre-pupae 48 hr post-infection with Cg2001H. (F) Quantification of ROS levels in non-infected, banana-fed and *C. glabrata* infected w^{1118};*drsGFP* larvae. (G) Percentage of larvae surviving to pupae in non-infected, banana fed and *C. glabrata* infected w^{1118};*drsGFP* larvae. Error bars represent the standard deviation of 3 independent biological experiments; ns $P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$ indicate significant values when compared to non-infected larvae.

RESULTS

The host response following C. glabrata GI infection

To establish an oral infection screen for a *C. glabrata* mutant library, we developed a third instar larval yeast-feeding protocol (see material and methods for details). Briefly, we fed larvae a *C. albicans* or *S. cerevisae* or *C. glabrata*/banana mixture while in parallel to each infection, larvae fed with banana only, served as our control. For these experiments we made use of third instar w^{1118} larvae (a wild type strain and the genetic background of the mutant fly strains used in this study). First, we determined the dynamics of host immune triggering after GI infection with reference yeast strains compared to the banana-only control (Figures 1A-C). As a measure of Toll pathway activity we monitored *drs* mRNA levels at specific time-points (Figure 1A-C). On average, 24h was the time point where the most elevated *drs* gene expression levels was seen (Figures 1A-C). However, there was a significant difference in the capacity of the three fungi to activate *drs* gene expression with *C. albicans*-induced activation almost 10x more compared to *S. cerevisiae* and *C. glabrata* (Figure 1A-C). Nevertheless, compared to non-infected larvae (Figure 1D), there was a robust fat body induction of a *drs-GFP* marker in larvae infected with the reference *C. glabrata* strain Cg2001H (Figure 1E). This confirmed a systemic response after feeding and was reminiscent of the same result in *C. albicans* infection [37]. In addition, there was significant increase in ROS production in Cg2001H-infected larvae over and above the banana control (Figure 1F). Finally there was a significantly reduced survival of GI infected larvae at 24h compared to both the banana control and to non-infected larvae (Figure 1G). Of note, compromised survival of w^{1118} was not an effect of that specific genetic background as it was also observed in $Oregon^{R}$ and w^{1118}; *drs-GFP* larvae (Figure S1, A and B in File S1).

Exploring individual strain infectivity in a library of C. glabrata deletion mutants

After determining that the host responses to GI infection following *C. albicans* and *C. glabrata* infection were comparable (albeit not of the same intensity), we used the system to screen a library of *C. glabrata* deletion mutants (TFKO library) for their ability to activate the Toll pathway. The library included deletions of 196 *C. glabrata* transcription factors (TF), with well-characterized roles in other yeast species (Schwarzmüller *et al.*, 2014). In this manner, we would be able to pinpoint to gene regulatory networks organized by specific transcription factors that are involved in infectivity. Table S1 presents all the strains used for the screen. We used w^{1118}; *drs-GFP* as a proxy for immune induction and virulence. Five of the 196 *C. glabrata* TF mutants (*Δada2*, *Δbas1*, *Δhir3*, *Δino2*, *Δmet31*) did not activate *drs* expression at 24h following infection (Figure 2A). To ascertain that this phenomenon was due to the deleted TFs, complementation strains were constructed. Complemented strains were able to activate *drs* to a degree that was statistically indistinguishable from the *C. glabrata*

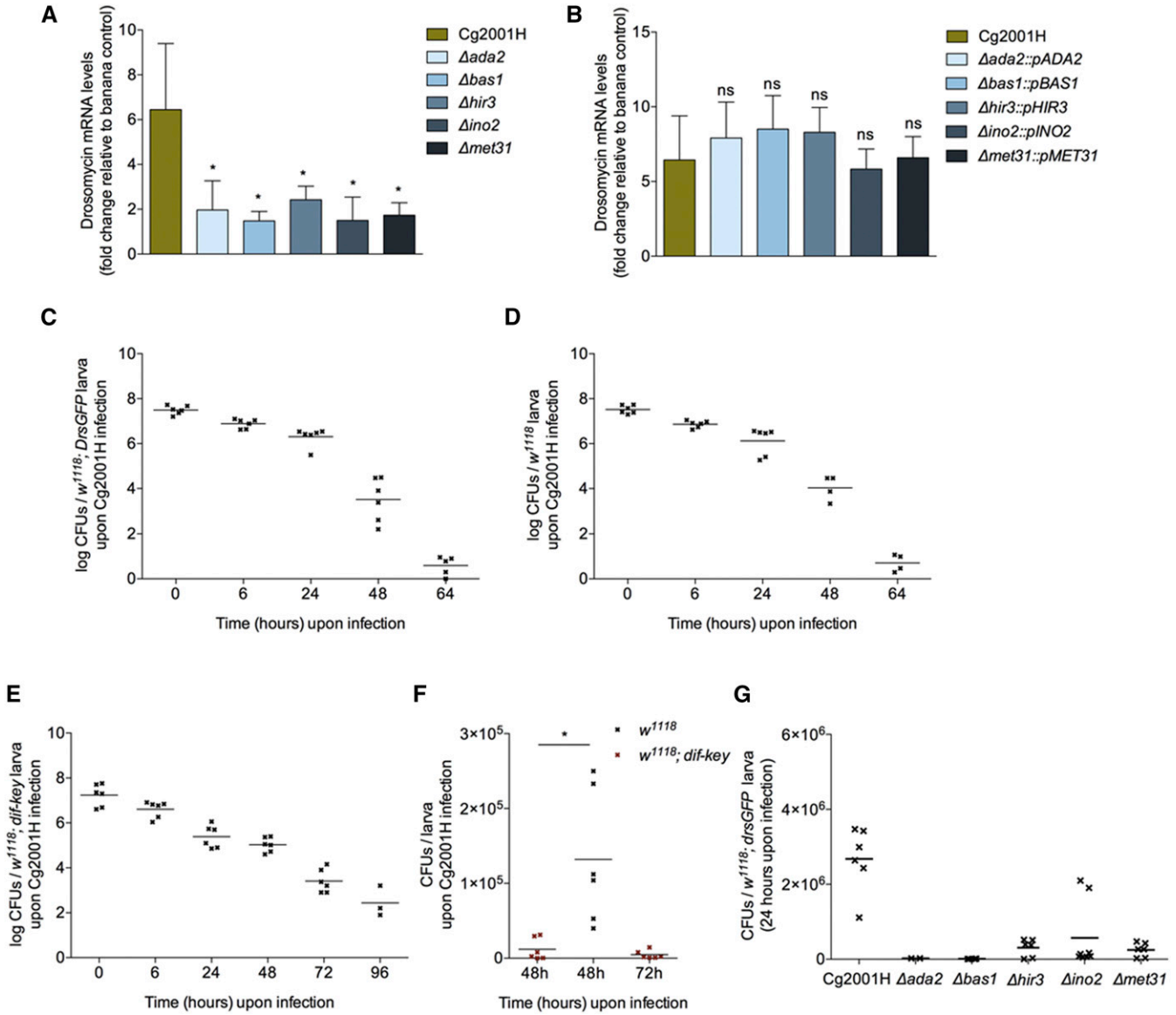

Figure 2 Gastrointestinal infection with deletion mutant strains. (A) *Drosomycin* mRNA gene expression levels relative to banana control in w^{1118}; *drsGFP* larvae and the TFKO deletion mutants (hit strains only), 24 hr following infection with Cg2001H. (B) Quantification of *drosomycin* gene expression following infection of w^{1118};*drsGFP* with Cg2001H and the corresponding complemented strains of the TFKO deletion mutants relative to banana control 24 hr post-infection. Error bars represent the standard deviation of 5 separate experiments; ns $P > 0.05$, $^{*}P < 0.05$ indicate significant values when compared to w^{1118};*drsGFP* infected with Cg2001H. CFUs/fly of *C. glabrata* in (C) w^{1118};*drsGFP* larvae, (D) w^{1118} larvae and (E) w^{1118};*dif-key* larvae at selected time points post-infection with Cg2001H. (F) CFUs/fly of Cg2001H in w^{1118} 48 hr post-infection and in w^{1118};*dif-key* 48 and 72 hr after infection. (G) CFUs/fly of Cg2001H and the 5 deletion mutants (Δ*ada2*, Δ*bas1*, Δ*hir3*, Δ*ino2* and Δ*met31*) in w^{1118}; *drsGFP* larvae 24 hr post-infection.

reference strain, *Cg2001H* (Figure 2B). In contrast, this was not the case when the TFKO strains were complemented with just the empty vector (Figure S2A in File S1; for a list of the complementation strains used see Table S1). We used Colony Forming Units (CFUs) to monitor the presence of *Cg2001H* inside GI infected larvae. W^{1118}; *drs-GFP* larvae presented similar kinetics with w^{1118} larvae (Figure 2C and Figure 2D respectively), with *Cg2001* cleared at approx. 64h post-feeding. In contrast, immune-deficient w^{1118}; *dif-key* larvae showed delayed clearance with a considerable CFUs of *Cg2001H* still at 96h post-feeding (Figure 2E). Direct comparison between w^{1118} and w^{1118}; *dif-key* larvae showed that at 48h post-infection, there was a significant difference in CFUs with a 24h difference in clearance (Figure 2F). Nevertheless, the five TFKO strains that did not activate *drs* in our screen were cleared significantly faster than *Cg2001H* with zero or much lower CFU count at 24h post-infection (Figure 2G).

Ex vivo phenotypic characterization of C. glabrata TF mutants failing to grow inside the host

To pinpoint a mechanism by which the *C. glabrata* mutants may be cleared in the larval host, the mutants were tested for growth on several conditions relating to the host environment. This included nutrient limitation tolerance, pH and temperature sensitivity and susceptibility to antifungals (Figure 3A; for a list of all *ex vivo* conditions tested see Table S2). *C. glabrata* Δ*bas1* and Δ*hir3* grew similarly to the parental

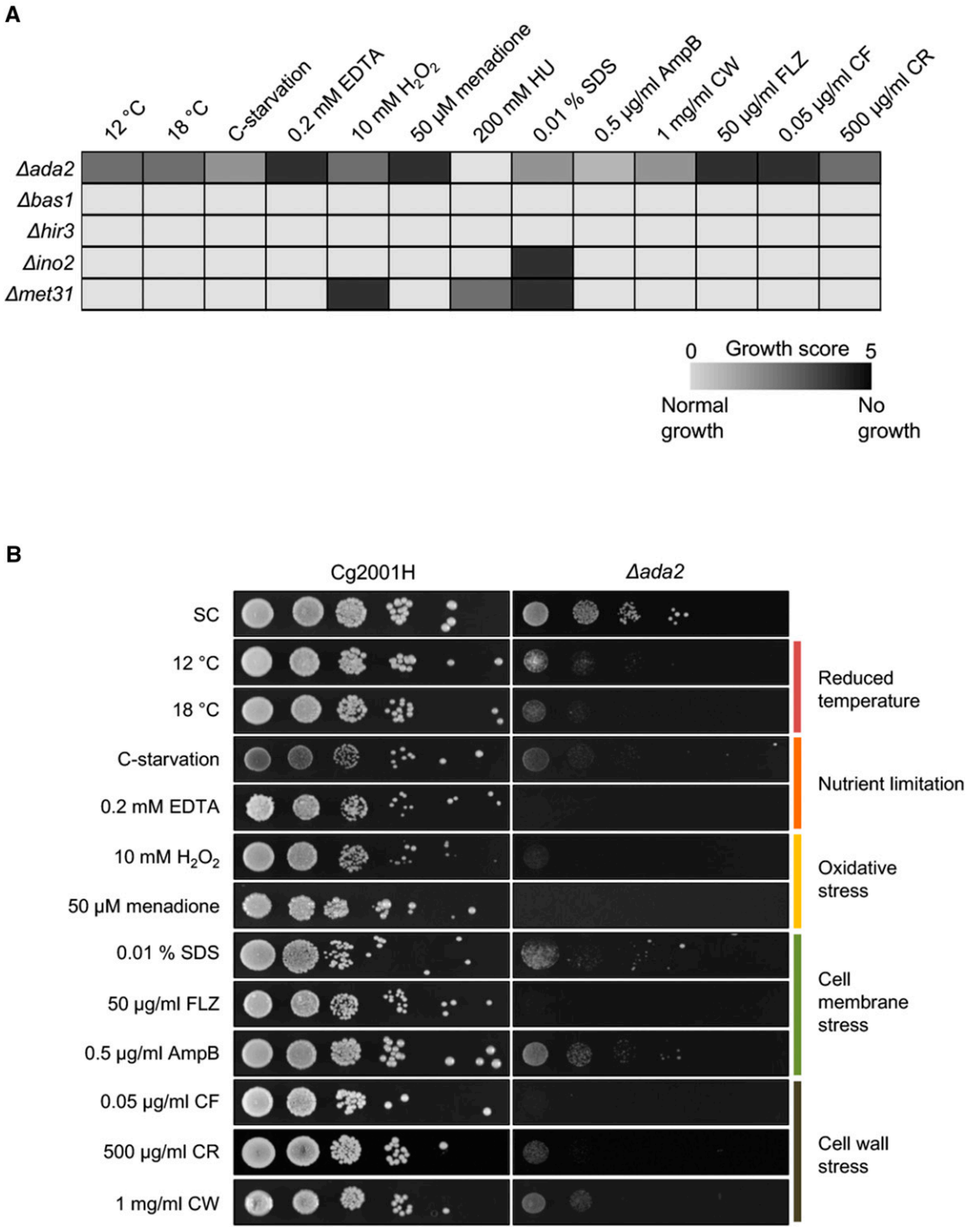

Figure 3 *Ex vivo* phenotypic growth of the deletion mutant strains. (A) Selected *C. glabrata* mutants were tested for growth under stress conditions targeting a variety of cellular processes and structures. Ten-fold serial dilutions of parental strain Cg2001H and deletion mutants Δ*ada2*, Δ*bas1*, Δ*hir3*, Δ*ino2* and Δ*met31* were spotted onto SC (synthetic complete) agar supplemented with the described chemical agents. Plates were incubated at 30°C and scored for growth. Stress conditions for which all strains showed normal growth are not included. HU = hydroxyurea, SDS = sodium dodecyl sulfate, AmpB = amphotericin B, CW = calcofluor white, FLZ = fluconazole, CF = caspofungin, CR = congo red. (B) Serial dilution images of Δada2 phenotypes relative to parental strain Cg2001H. Stress conditions are grouped according stress type and targets.

strain Cg2001 under all stress conditions tested while Δ*ino2* was susceptible to 0.01%SDS (Figure 3A). In addition, Δ*met31* was susceptible to H_2O_2, 20mM HU and 0.01% SDS (Figure 3A). The most susceptible phenotypes were observed for *C. glabrata* Δ*ada2* which was susceptible to growth on 10 out of 13 of the tested conditions relating to growth under decreased temperatures, nutrient limitation, oxidative stress, cell membrane stress, cell wall stress and antifungal drug tolerance (Figure 3B, Table S3).

The five TF deletion mutants were further investigated for any possible dysfunctions, which may relate to their reduced ability to persist in the larval host. Initially, we monitored growth under non-stressed conditions at 30°. Outside the host, all mutant strains displayed growth curves comparable to the reference strain (Figure S3A in File S1). These growth rate measurements indicated that these TF mutants were not growth-defective. However, *C. glabrata* Δ*ada2* and Δ*bas1* deletion mutants displayed an increased (longer) generation time (Figure S3B in File S1).

The susceptibility of *C. glabrata* Δ*ada2* to oxidative stress inducing agents H_2O_2 and menadione were of particular interest since increased levels of ROS were recorded upon *Cg2001H* infection in earlier experiments (Figure 1F). Therefore, we explored the hypothesis that ADA2 was indispensable for resistance to toxic levels of oxidative stress, something that would be the case when encountering the localized epithelial immune response of the *Drosophila* GI tract.

The role of the C. glabrata TF ADA2 in Drosophila GI infection

The *Drosophila* dual oxidase (dDuox) has been shown to regulate ROS in the fly intestine (Ha *et al.* 2005). When *dDuox* was knocked down in enterocytes (w^{1118}; *np1-GAL4*; $Duox^{RNAi}$), larval survival following GI infection with *Cg2001H* was significantly reduced (Figure 4A). This showed that absence of ROS production increased susceptibility of larvae to GI infection. In contrast, survival of these larvae following infection by the *Δada2* deletion mutant was significantly improved compared to *Cg2001H* (Figure 4A). This showed that when both host ROS production as well as pathogen ROS defenses were absent larval survival was largely restored. This was not the case when *Δada2* was complemented with a wild type copy of the *ada2* gene (Figure 4A). Consistent with the role of *ada2* in the process, complementation of the *Δada2* mutant with an empty vector did not compromise larval survival (Figure 4A). Finally, adding an extra copy of the *ada2* gene in the *Cg2001H* reference strain made the latter more virulent than normal when host ROS production was supressed (Figure 4A). This underlined the role of *ada2* as a major regulator of *C. glabrata* virulence beyond ROS defenses in the context of gastrointestinal infection. In contrast, none of the other mutants compromised larval survival when overexpressed (Figure 4B).

The above increased pathogenicity was further confirmed in various strains like the GAL4 and RNAi lines used to supress ROS production

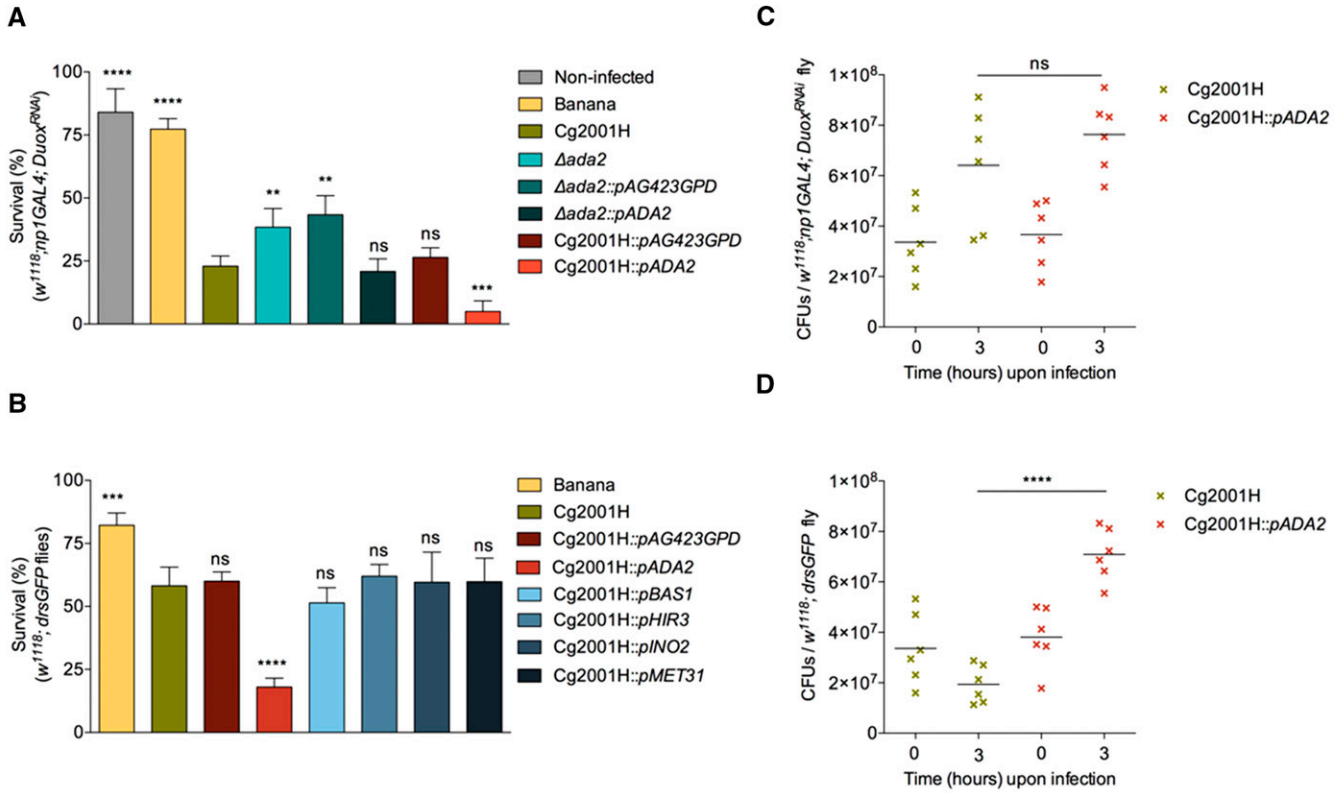

Figure 4 Oral infection of ROS suppressed larvae (A) The percentage of ROS suppressed larvae developing into pupae in non-infected, banana fed larvae, or larvae infected by various strains including Cg2001H, Δ*ada2* deletion strain, Δ*ada2* deletion strain with empty vector (Δ*ada2*::*pAG423GPD*), Δ*ada2* complement strain (Δ*ada2*::*pADA2*), wild type *C. glabrata* with empty vector (Cg2001H::*pAG423GPD*) and *ada2* overexpression strain (Cg2001H::*pADA2*). (B) The percentage of w^{1118};*drsGFP* larvae developed into pupae in banana-fed larvae, infected larvae with wild type Cg2001H, *C. glabrata* with empty vector (Cg2001H::*pAG423GPD*) and *ada2*, *bas1*, *hir2*, *ino2* and *met31* overexpression strains (C) CFUs/fly 0 and 3 hr after infection with Cg2001H and *ada2* overexpression strain in ROS suppressed larvae (w^{1118}; *np1GAL4*; $Duox^{RNAi}$). (D) CFUs/fly 0 and 3 hr after infection with Cg2001H and *ada2* overexpression strain in w^{1118} larvae; ns $P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$ indicate significant values when compared to larvae infected with Cg2001H.

(Figure S4, A and B in File S1 respectively), w^{1118} (as the genetic background of all of the mutants used; Figure S4C in File S1) and $Oregon^R$ flies (as an extra genetic background; Figure S4D in File S1). Overexpression strains of the rest of the hits were also constructed (Table S1) and their pathogenicity was examined in the same host background like the *ada2* overexpression strain. Survival of larvae showed that hyper virulence of *ada2* overexpression was unique among the other TFKO hits of the original screen (Figure 4C).

CFU measurements revealed that there was an expansion of both *Cg2001H* and *Cg2001H* overexpressing *ada2* (*Cg2001H::pADA2*) when *Duox* gene expression was knocked-down (Figure 4C) resulting in increased larvae lethality. However, in case of infection of a wild-type host strain (with normal ROS production) there was a significant increase in CFUS of *Cg2001H::pADA2* in contrast to the clearance of the reference *Cg2001H* strain (Figure 2C and Figure 4D). This meant that an extra copy of *ada2* allowed increased growth of the pathogen. Moreover, as seen by the larval survival assays, elevated growth of *Cg2001H::pADA2* compromised the host significantly more than the wild type *C. glabrata*. However, this growth advantage was lost when larvae were unable to produce ROS. Taken together with the inability of *ada2* to grow in even the lowest of H_2O_2 concentrations, the above results suggest that the TF *ada2* is important for regulating *C. glabrata* defenses against host-generated ROS.

DISCUSSION

C. glabrata is becoming an important problem in persistent hospital infections (Pfaller and Diekema 2007). Starting as a commensal microbe it can generate systemic challenges that have an increasingly incurable outlook (Kaur *et al.*, 2007). The growing resistance of *C. glabrata* to various antifungals including azoles underlines the above and indicates the need to develop new drugs and/or therapies to target this pathogen (Perlin 2007; Pfaller *et al.*, 2010; Perlin 2014; Singh-Babak *et al.* 2012). To this end, we screened a *C. glabrata* mutant library of transcription factor and co-factor deletions (TFKO library, see Schwarzmüller *et al.*, 2014) to identify possible novel virulence factors responsible for *C. glabrata* pathogenicity in the GI tract. Five candidate genes were identified and their ability to infect the host was explored further. Five deletion strains showed accelerated clearance by the host gut. Considering that gut conditions are a new stress environment for the pathogen we followed a series of *ex vivo* stress response tests including oxidative stress sensitivity, antifungal sensitivity, pH and temperature sensitivity. Between the five deletion mutant hits, Δ*ada2* deletion showed severe growing limitations under oxidative stress response tests. Therefore, this particular mutant was studied further. In addition, *ada2* deficient *C. glabrata* was unable to activate a robust systemic immune response. This was either due to the fact that the mutation altered the cell wall in a manner rendered unrecognizable by GNBP3 or because proteases normally secreted and able to activate PSH directly were not upregulated. More work on the re-wiring of transcription of wild type *C. glabrata* and *ada2* mutants within the host is needed to distinguish between these two possibilities.

The side of the host

The synthesis of ROS (including H_2O_2, hydroxyl radicals and superoxide) as an immune defense is the first line immune response by phagocytes upon encountering a fungal infection in humans. Similarly, the production of both ROS and AMPs are important features of *Drosophila* epithelial immunity (Tzou *et al.*, 2000; Ha *et al.*, 2005; Buchon *et al.*, 2009). ROS can cause damage to DNA, RNA, and proteins and trigger the oxidative degradation of lipids in cell membranes. GI infection of *Drosophila* larvae by *C. glabrata* increased ROS levels significantly when compared to non-infected or banana-only treated controls. When the percentage of larvae surviving to pupae was determined, we found that although feeding with banana activated a low level ROS induction (Figure 1F) this did not affect the survival of the organism. However, infection with the reference strain *Cg2001H* did impact survival with approx. 60–65% of larvae developing to pupae. Oral infection with *C. glabrata* activated epithelial immunity in the GI tract by inducing both ROS production as well as leading to a systemic activation of the Toll pathway. Lack of the Toll and Imd pathways delayed but did not stop the clearance of the pathogen, implying a major role for ROS in the equilibrium between host and pathogen. The crucial role of ROS was further supported when we recorded significantly increased levels of host lethality in larvae that were unable to produce ROS.

The side of the pathogen

Five deletion mutants from the TFKO library showed accelerated clearance and inability to induce an immune response inside the host. Growth curves of the 5 deletion mutants showed that all the mutants were able to successfully grow at 30° in YPD media, outside of the host. Moreover, the generation times for the 5 deletion mutants were similar, with Δ*ada2* and Δ*bas1* displaying a marginally shorter generation time than the other strains. Nonetheless, all the deletion strains did not display any detectable growth defects that would be the cause of its rapid elimination from the host. The ability of the deletion mutants to grow under different stress conditions included tests for nutrient limitation tolerance, sensitivity to antifungals, defects in cell wall/membrane and sensitivity to oxidative stress conditions, for example. All but one strain were able to grow under all the conditions, albeit reduced and limited growth was observed. However, Δ*ada2* deletion mutant showed high sensitivity to the oxidative stress conditions with very restricted or no growth. As previously mentioned, wild type *C. glabrata* has shown to grow in extremely high H_2O_2 conditions (Cuéllar-Cruz *et al.*, 2008). Thus, the inability of Δ*ada2* mutants to grow in low H_2O_2 conditions indicated that the *ada2* gene is essential for its resistance to the ROS-mediated immune defense in the *Drosophila* gastrointestinal tract. To this direction, Δ*ada2* mutant was investigated further using ROS-suppressed flies.

Δada2 mutant and the interaction with host-generated ROS

The open reading frame (ORF) of *ada2* in *C. glabrata* is CAGL0K06193g and is known to be involved in the transcriptional activation of RNA polymerase II. Moreover, ortholog(s) are also implicated in chromatin binding and histone acetyltransferase. This suggests that the gene function of *ada2* is very broad and is likely to be involved in the activation of various proteins. Indeed, genome-wide mapping of ADA complexes in *Candida albicans* have shown that ADA2 is recruited to 200 promoters of genes involved in different stress-response functions (including oxidative resistance) and metabolic responses (Sellam *et al.* 2009). Consistent with these results, our ex *vivo* experiments clearly showed that Δ*ada2* deletion mutants were highly sensitive to various oxidative stress conditions suggesting that among the many functions of this gene is also the co-ordination of oxidative stress coming from the ROS-producing host immune reaction. In *C. albicans*, host-derived ROS affects only fungal cells exposed to phagocytosis and epithelial immunity rather than those in systemic infection (Enjalbert *et al.*, 2007). Mechanisms to avoid or defend against host-derived ROS in this fungus include extracellular anti-oxidant enzymes of the super dismutase (SOD) family of

enzymes (Frohner *et al.*, 2009; Hwang *et al.*, 2002) all involving SAGA/ADA-mediated co-activation (Sellam *et al.* 2009). Both its relation with ROS stress resistance and the fact that in *C. albicans* ADA2p binds the promoters of several drug transporters (*mdr1*, *cdr1*, *cdr4*, *qdr1*, *ycf1*, *flu1* to name a few) makes ADA2 an important target for the development of new antifungals if one wanted to eliminate *C. glabrata* from the GI tract (Sellam *et al.*, 2009) (see below).

When orally infected with Δ*ada2* mutants, larvae with supressed ROS production were able to survive like wild type controls, indicating that the inability of the gut to synthesize ROS had no impact on host surviving under these conditions. However, infection with the wild type *C. glabrata Cg2001H* strain resulted in a significant decrease in survival of the same larvae. This indicated that in the absence of the *ada2* gene, ROS-suppressed larvae were able to survive better. When *ada2* gene was re-introduced, virulence was restored. Moreover, when this gene was overexpressed, survival of ROS suppressed flies decreased significantly when compared to infections with the *Cg2001H* strain. Finally, in wild type (w^{1118}) larvae with functioning Duox, *ada2* overexpression strain was able to persist and expand three hours after infection while the fungal load of wild type *C. glabrata* decreased. This indicated that an additional copy of the *ada2* gene made this strain hyper-virulent, significantly reducing the survival of the host and enabling expansion within the host. Contrastingly, survival of w^{1118}; *drsGFP* larvae when infected with the remaining 4 overexpression strains of the deletion mutant hits (Δ*bas1*, Δ*hir3*, Δ*ino2* and Δ*met31*) was similar to wild type *C. glabrata* highlighting the specific effect of *ada2* overexpression.

The induction of ROS has been shown to be a rapid and important immune defense following oral infection in *Drosophila* (Ha *et al.*, 2005). Flies that lack the ability to generate ROS upon natural infection succumb to infection. Ha and colleagues (Ha *et al.*, 2005) demonstrated ubiquitous expression of *Duox-RNAi* resulted in increased mortality following natural infection with *Ecc15*. Likewise, when *Duox-RNAi* was restricted to the gut, flies displayed a similar level of mortality. Interestingly, when *Duox-RNAi* was introduced to the main immune tissues in systemic immunity in *Drosophila* (the fat body/hemocytes), survival of flies was unaffected (Ha *et al.*, 2005). This indicates that ROS plays a major role in the host resistance during GI infection. Therefore, the necessity for ADA2-mediated transcription to resist host-generated ROS as implied by our results may be a gut-specific interaction.

Indeed, a recent study has indicated that although an independently constructed *ada2* mutant exhibited susceptibility to three classes of antifungal drugs (*i.e.*, azoles, echinocandins, and polyenes) as well as cell-wall perturbing agents it was hyper-virulent in a mouse model of systemic infection (Yu *et al.*, 2018). This was contrary to what we have observed in our *Drosophila* model. However, since the route of infection was different our hypothesis is that GI *vs.* systemic immunity may influence the virulence outcome of *ada2* mutants. More work is needed to determine the difference in the interaction between *ada2* mutants with the various aspects of host defense.

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LITERATURE CITED

Alarco, A. M., A. Marcil, J. Chen, B. Suter, D. Thomas *et al.*, 2004 Immune-deficient *Drosophila melanogaster*: a model for the innate immune response to human fungal pathogens. J. Immunol. 172(9): 5622–5628. https://doi.org/10.4049/jimmunol.172.9.5622

Albrecht, A., A. Felk, I. Pichova, J. R. Naglik, M. Schaller *et al.*, 2006 Glycosylphosphatidylinositol-anchored proteases of *Candida albicans* target proteins necessary for both cellular processes and host-pathogen interactions. J. Biol. Chem. 281(2): 688–694. https://doi.org/10.1074/jbc.M509297200

Basset, A., R. S. Khush, A. Braun, L. Gardan, F. Boccard *et al.*, 2000 The phytopathogenic bacteria *Erwinia carotovora* infects Drosophila and activates an immune response. Proc. Natl. Acad. Sci. USA 97(7): 3376–3381. https://doi.org/10.1073/pnas.97.7.3376

Brown, A. J., K. Haynes, and J. Quinn, 2009 Nitrosative and oxidative stress responses in fungal pathogenicity. Curr. Opin. Microbiol. 12(4): 384–391. https://doi.org/10.1016/j.mib.2009.06.007

Brunke, S., J. Quintin, L. Kasper, I. D. Jacobsen, M. E. Richter *et al.*, 2015 Of mice, flies–and men? Comparing fungal infection models for large-scale screening efforts. Dis. Model. Mech. 8(5): 473–486. https://doi.org/10.1242/dmm.019901

Buchon, N., N. A. Broderick, M. Poidevin, S. Pradervand, and B. Lemaitre, 2009 Drosophila intestinal response to bacterial infection: activation of host defense and stem cell proliferation. Cell Host Microbe 5(2): 200–211. https://doi.org/10.1016/j.chom.2009.01.003

Butler, G., D. Matthew, M. D. Rasmussen, M. F. Lin, M. A. Santos *et al.*, 2009 Evolution of pathogenicity and sexual reproduction in eight *Candida* genomes. Nature 459(7247): 657–662. https://doi.org/10.1038/nature08064

Chamilos, G., M. S. Lionakis, R. E. Lewis, J. L. Lopez-Ribot, S. P. Saville *et al.*, 2006 *Drosophila melanogaster* as a facile model for large-scale studies of virulence mechanisms and antifungal drug efficacy in Candida species. J. Infect. Dis. 193(7): 1014–1022. https://doi.org/10.1086/500950

Cormack, B. P., N. Ghori, and S. Falkow, 1999 An adhesin of the yeast pathogen *Candida glabrata* mediating adherence to human epithelial cells. Science 285(5427): 578–582. https://doi.org/10.1126/science.285.5427.578

Csank, C., and K. Haynes, 2000 *Candida glabrata* displays pseudohyphal growth. FEMS Microbiol. Lett. 189(1): 115–120. https://doi.org/10.1111/j.1574-6968.2000.tb09216.x

Cuéllar-Cruz, M., M. Briones-Martin-del-Campo, I. Cañas-Villamar, J. Montalvo-Arredondo, L. Riego-Ruiz *et al.*, 2008 High resistance to oxidative stress in the fungal pathogen *candida glabrata* is mediated by a single catalase, Cta1p, and is controlled by the transcription factors Yap1p, Skn7p, Msn2p, and Msn4p. Eukaryot. Cell 7(5): 814–825. https://doi.org/10.1128/EC.00011-08

Dujon, B., D. Sherman, G. Fischer, P. Durrens, S. Casaregola *et al.*, 2004 Genome evolution in yeasts. Nature 430(6995): 35–44. https://doi.org/10.1038/nature02579

Enjalbert, B., D. M. MacCallum, F. C. Odds, and A. J. Brown, 2007 Niche-specific activation of the oxidative stress response by the pathogenic fungus *Candida albicans*. Infect. Immun. 75(5): 2143–2151. https://doi.org/10.1128/IAI.01680-06

Fang, F. C., 2004 Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. Nat. Rev. Microbiol. 2(10): 820–832. https://doi.org/10.1038/nrmicro1004

Ferrandon, D., A. C. Jung, M. Criqui, B. Lemaitre, S. Uttenweiler-Joseph *et al.*, 1998 A drosomycin-GFP reporter transgene reveals a local immune response in Drosophila that is not dependent on the Toll pathway. EMBO J. 17(5): 1217–1227. https://doi.org/10.1093/emboj/17.5.1217

Frohner, I. E., C. Bourgeois, K. Yatsyk, O. Majer, and K. Kuchler, 2009 *Candida albicans* cell surface superoxide dismutases degrade host-derived reactive oxygen species to escape innate immune surveillance. Mol. Microbiol. 71(1): 240–252. https://doi.org/10.1111/j.1365-2958.2008.06528.x

Ghannoum, M. A., 2000 Potential role of phospholipases in virulence and fungal pathogenesis. Clin. Microbiol. Rev. 13(1): 122–143. https://doi.org/10.1128/CMR.13.1.122-143.2000

Glittenberg, M. T., S. Silas, D. M. MacCallum, N. A. Gow, and P. Ligoxygakis, 2011a Wild-type *Drosophila melanogaster* as an alternative model system for investigating the pathogenicity of *Candida albicans*. Dis. Model. Mech. 4(4): 504–514. https://doi.org/10.1242/dmm.006619

Glittenberg, M. T., I. Kounatidis, D. Christensen, M. Kostov, S. Kimber *et al.*, 2011b Pathogen and host factors are needed to provoke a systemic host response to gastrointestinal infection of Drosophila larvae by *Candida albicans*. Dis. Model. Mech. 4(4): 515–525. https://doi.org/10.1242/dmm.006627

Gottar, M., V. Gobert, A. A. Matskevich, J-M. Reichhart, C Wang *et al.*, 2006 Dual detection of fungal infections in Drosophila via recognition of glucans and sensing of virulence factors. Cell 29;127(7): 1425–37. https://doi.org/10.1016/j.cell.2006.10.046

Gow, N. A., F. L. Van de Veerdonk, A. J. Brown, and M. G. Netea, 2011 *Candida albicans* morphogenesis and host defence: discriminating invasion from colonization. Nat. Rev. Microbiol. 10(2): 112–122. https://doi.org/10.1038/nrmicro2711

Ha, E. M., C. T. Oh, Y. S. Bae, and W. J. Lee, 2005 A direct role for dual oxidase in Drosophila gut immunity. Science 310(5749): 847–850. https://doi.org/10.1126/science.1117311

Hwang, C. S., G. E. Rhie, J. H. Oh, W. K. Huh, H. S. Yim *et al.*, 2002 Copper- and zinc-containing superoxide dismutase (Cu/ZnSOD) is required for the protection of *Candida albicans* against oxidative stresses and the expression of its full virulence. Microbiology 148(11): 3705–3713. https://doi.org/10.1099/00221287-148-11-3705

Jacobsen, I. D., K. Grosse, A. Berndt, and B. Hube, 2011 Pathogenesis of *Candida albicans* infections in the alternative chorio-allantoic membrane chicken embryo model resembles systemic murine infections. PLoS One 6(5): e19741 (erratum: PLoS One 6(6): 10.1371/annotation/1912450a-b5a7-4dbe-bc2a-cd93579fb0dd). https://doi.org/10.1371/journal.pone.0019741

Jacobsen, I. D., S. Brunke, K. Seider, T. Schwarzmüller, A. Firon *et al.*, 2010 *Candida glabrata* persistence in mice does not depend on host immunosuppression and is unaffected by fungal amino acid auxotrophy. Infect. Immun. 78(3): 1066–1077. https://doi.org/10.1128/IAI.01244-09

Kaloriti, D., A. Tillmann, E. Cook, M. Jacobsen, T. You *et al.*, 2012 Combinatorial stresses kill pathogenic Candida species. Med. Mycol. 50(7): 699–709. https://doi.org/10.3109/13693786.2012.672770

Kaur, R., R. Domergue, M. L. Zupancic, and B. P. Cormack, 2005 A yeast by any other name: *Candida glabrata* and its interaction with the host. Curr. Opin. Microbiol. 8(4): 378–384. https://doi.org/10.1016/j.mib.2005.06.012

Kaur, R., B. Ma, and B. P. Cormack, 2007 A family of glycosylphosphatidylinositol-linked aspartyl proteases is required for virulence of *Candida glabrata*. Proc. Natl. Acad. Sci. USA 104(18): 7628–7633. https://doi.org/10.1073/pnas.0611195104

Kounatidis, I., and P. Ligoxygakis, 2012 Drosophila as a model system to unravel the layers of innate immunity to infection. Open Biol. 2(5): 120075. https://doi.org/10.1098/rsob.120075

Lemaitre, B., E. Nicolas, L. Michaut, J. M. Reichhart, and J. A. Hoffmann, 1996 The dorsoventral regulatory gene cassette spätzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell 86(6): 973–983. https://doi.org/10.1016/S0092-8674(00)80172-5

Li, L., H. Kashleva, and A. Dongari-Bagtzoglou, 2007 Cytotoxic and cytokine-inducing properties of Candida glabrata in single and mixed oral infection models. Microb. Pathog. 42(4): 138–147. https://doi.org/10.1016/j.micpath.2006.12.003

Massey, S. E., G. Moura, P. Beltrão, R. Almeida, J. R. Garey *et al.*, 2003 Comparative evolutionary genomics unveils the molecular mechanism of reassignment of the CTG Codon in *Candida* spp. Genome Res. 13(4): 544–557. https://doi.org/10.1101/gr.811003

Méan, M., O. Marchetti, and T. Calandra, 2008 Bench-to-bedside review: Candida infections in the intensive care unit. Crit. Care 12(1): 204. https://doi.org/10.1186/cc6212

Paquette, N., M. Broemer, K. Aggarwal, L. Chen, M. Husson *et al.*, 2010 Caspase Mediated Cleavage, IAP Binding and Ubiquitination: Linking Three Mechanisms Crucial for Drosophila NF-κB Signaling. Mol. Cell 37(2): 172–182. https://doi.org/10.1016/j.molcel.2009.12.036

Perlin, D., 2014 Current perspectives on echinocandin class drugs. Future Microbiol. 6(4): 441–457. https://doi.org/10.2217/fmb.11.19

Perlin, D. S., 2007 Resistance to echinocandin-class antifungal drugs. Drug Resist. Updat. 10(3): 121–130. https://doi.org/10.1016/j.drup.2007.04.002

Perlroth, J., B. Choi, and B. Spellberg, 2007 Nosocomial fungal infections: epidemiology, diagnosis, and treatment. Med. Mycol. 45(4): 321–346. https://doi.org/10.1080/13693780701218689

Pfaller, M. A., and D. J. Diekema, 2007 Epidemiology of invasive candidiasis: a persistent public health problem. Clin. Microbiol. Rev. 20(1): 133–163. https://doi.org/10.1128/CMR.00029-06

Pfaller, M. A., D. J. Diekema, D. L. Gibbs, V. A. Newell, R. Barton *et al.*, 2010 Geographic variation in the frequency of isolation and fluconazole and voriconazole susceptibilities of *Candida glabrata*: An assessment from the ARTEMIS DISK Global Antifungal Surveillance Program. Diagn. Microbiol. Infect. Dis. 67(2): 162–171. https://doi.org/10.1016/j.diagmicrobio.2010.01.002

Roetzer, A., T. Gabaldón, and C. Schüller, 2011 From *Saccharomyces cerevisiae* to *Candida glabrata* in a few easy steps: Important adaptations for an opportunistic pathogen. FEMS Microbiol. Lett. 314(1): 1–9. https://doi.org/10.1111/j.1574-6968.2010.02102.x

Rutschmann, S., A. C. Jung, C. Hetru, J. M. Reichhart, J. A. Hoffmann *et al.*, 2000 The Rel protein DIF mediates the antifungal but not the antibacterial host defence in Drosophila. Immunity 12(5): 569–580. https://doi.org/10.1016/S1074-7613(00)80208-3

Rutschmann, S., A. Kilinc, and D. Ferrandon, 2002 The Toll pathway is required for resistance to Gram-positive bacterial infections in Drosophila. J. Immunol. 168(4): 1542–1546. https://doi.org/10.4049/jimmunol.168.4.1542

Sellam, A., C. Askew, E. Epp, H. Lavoie, M. Whiteway *et al.*, 2009 Genome-wide mapping of the co-activator Ada2p yields insight into the functional roles of SAGA/ADA complex in *Candida albicans*. Mol. Biol. Cell 20(9): 2389–2400. https://doi.org/10.1091/mbc.E08-11-1093

Saijo, S., T. Miyazaki, K. Izumikawa, T. Mihara, T. Takazono *et al.*, 2010 Skpn7p is involved in oxidative stress response and virulence of *Candida glabrata*. Mycopathologia 169(2): 81–90. https://doi.org/10.1007/s11046-009-9233-5

Schmittgen, T. D., and K. J. Livak, 2008 Analyzing real-time PCR data by the comparative CT method. Nat. Protoc. 3(6): 1101–1108. https://doi.org/10.1038/nprot.2008.73

Schwarzmüller, T., B. Ma, E. Hiller, F. Istel, M. Tscherner *et al.*, 2014 Systematic phenotyping of a large-scale *Candida glabrata* deletion collection reveals novel antifungal tolerance genes. PLoS Pathog. 10(6): e1004211. https://doi.org/10.1371/journal.ppat.1004211

Seider, K., S. Brunke, L. Schild, D. Wilson, O. Majer *et al.*, 2011 The facultative intracellular pathogen *Candida glabrata* subverts macrophage cytokine production and phagolysosome maturation. J. Immunol. 187(6): 3072–3086. https://doi.org/10.4049/jimmunol.1003730

Singh-Babak, S. D., T. Babak, S. Diezmann, J. A. Hill, J. L. Xie *et al.*, 2012 Global analysis of the evolution and mechanism of echinocandin resistance in *Candida glabrata*. PLoS Pathog. 8(5): e1002718. https://doi.org/10.1371/journal.ppat.1002718

Stöven, S., I. Ando, L. Kadalayil, Y. Engström, and D. Hultmark, 2000 Activation of the Drosophila NF-kappaB factor Relish by rapid endoproteolytic cleavage. EMBO Rep. 1(4): 347–352. https://doi.org/10.1093/embo-reports/kvd072

Thorne, T. W., H. Ho, M. Huvet, K. Haynes, and M. P. H. Stumpf, 2011 Prediction of putative protein interactions through evolutionary analysis of osmotic stress response in the model yeast *Saccharomyces* cerevisiae. Fungal Genet. Biol. 48(5): 504–511. https://doi.org/10.1016/j.fgb.2010.12.005

Tsoni, S. V., A. M. Kerrigan, M. J. Marakalala, N. Srinivasan, M. Duffield *et al.*, 2009 Complement C3 plays an essential role in the control of opportunistic fungal infections. Infect. Immun. 77(9): 3679–3685. https://doi.org/10.1128/IAI.00233-09

Tzou, P., S. Ohresser, D. Ferrandon, M. Capovilla, J. M. Reichhart *et al.*, 2000 Tissue-specific inducible expression of antimicrobial peptide genes in Drosophila surface epithelia. Immunity 13(5): 737–748. https://doi.org/10.1016/S1074-7613(00)00072-8

Wächtler, B., F. Citiulo, N. Jablonowski, S. Förster, F. Dalle *et al.*, 2012 *Candida albicans*-epithelial interactions: Dissecting the roles of active penetration, induced endocytosis and host factors on the infection process. PLoS One 7(5): e36952. https://doi.org/10.1371/journal.pone.0036952

Westwater, C., D. A. Schofield, P. J. Nicholas, E. E. Paulling, and E. Balish, 2007 *Candida glabrata* and *Candida albicans*; dissimilar tissue tropism and infectivity in a gnotobiotic model of mucosal candidiasis. FEMS Immunol. Med. Microbiol. 51(1): 134–139. https://doi.org/10.1111/j.1574-695X.2007.00287.x

Yu, S.-J., Y.-L. Chang, and Y.-L. Chen, 2018 Deletion of *ADA2* increases antifungal drug susceptibility and virulence in *Candida glabrata*. Antimicrob. Agents Chemother. 62(3): e01924–17. https://doi.org/10.1128/AAC.01924-17

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