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RESEARCH ARTICLE

Of mice, flies – and men? Comparing fungal infection models for large-scale screening efforts

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ABSTRACT

Studying infectious diseases requires suitable hosts for experimental in vivo infections. Recent years have seen the advent of many alternatives to murine infection models. However, the use of non-mammalian models is still controversial because it is often unclear how well findings from these systems predict virulence potential in humans or other mammals. Here, we compare the commonly used models, fruit fly and mouse (representing invertebrate and mammalian hosts), for their similarities and degree of correlation upon infection with a library of mutants of an important fungal pathogen, the yeast Candida glabrata.

Using two indices, for fly survival time and for mouse fungal burden in mutants of an important fungal pathogen, the yeast Candida glabrata, we show a good agreement between the models. Using two indices, for fly survival time and for mouse fungal burden in mutants of an important fungal pathogen, the yeast Candida glabrata, we show a good agreement between the models. We propose a suitable predictive model for estimating the virulence potential of C. glabrata mutants in the mouse from fly survival data. As examples, we found cell wall integrity mutants attenuated in flies, and mutants of a MAP kinase pathway had defective virulence in flies and reduced relative pathogen fitness in mice. In addition, mutants with strongly reduced in vitro growth generally, but not always, had reduced virulence in flies. Overall, we demonstrate that surveying Drosophila survival after infection is a suitable model to predict the outcome of murine infections, especially for severely attenuated C. glabrata mutants. Pre-screening of mutants in an invertebrate Drosophila model can, thus, provide a good estimate of the probability of finding a strain with reduced microbial burden in the mouse host.

KEY WORDS: Candida glabrata, Mutant library, Drosophila melanogaster, Alternative infection models, Signature-tagged mutagenesis, Fungal virulence factors

INTRODUCTION

The selection of suitable models is crucial in infection biology research. Deciding on the right model system for the biological question at hand requires deliberate weighing of many parameters, such as cost, amount of labor involved, throughput rate, degree of similarity to the human host, and ethical considerations. For example, the use of comparatively simple in vitro models when screening for novel antimicrobial drug candidates and investigating putative virulence factors in microbial pathogens is well established. However, in vivo models are still indispensable to provide the link between a gene and the clinically relevant outcome – disease or death of the host. Many different models – vertebrate and invertebrate – have been described in the past for the investigation of microbial virulence. Generally, using a murine model is considered the gold standard for most infections, due to its comparably high similarity to humans in terms of metabolism, body temperature, and immune system functions. Yet, working with mice requires specialized personnel, poses many practical difficulties, is often expensive and time consuming, and requires specific ethical considerations. Alternative in vivo infection models therefore, used ever more frequently. For pathogenic fungi, for example, these include – in no specific order: the vertebrate zebrafish model (Chao et al., 2010; Tobin et al., 2012), the embryonated chicken egg model (Jacobsen et al., 2011, 2010b), the nematode Caenorhabditis elegans (Breger et al., 2007), the insect models Galleria mellonella (Cotter et al., 2000; Jacobsen, 2014) and Drosophila melanogaster (Glittenberg et al., 2011; Limmer et al., 2011; Roetzer et al., 2008), and some more specialized models like Acanthamoeba spp. or Dictyostelium discoideum (Mylonakis et al., 2007; Steenbergen et al., 2001). Especially for large-scale screening efforts, for example, with libraries of hundreds or thousands of mutants, the benefits of using these systems are evident: they are generally easier to handle and, in contrast to mice, the use of hundreds of flies or worms for infection experiments is considered ethically acceptable.

Unfortunately, limitations remain associated with these non-mammalian infection systems. First and foremost, the body temperature of the non-mammalian hosts is generally significantly lower than that of humans. Indeed, many microbial virulence factors are expressed only, or more readily, at human body temperature. An important example is the morphological transition between yeasts and hyphae by Candida albicans, which is generally considered essential for full virulence of this fungus (Jacobsen et al., 2012) and which is induced by growth at 37°C. However, hyphae formation of C. albicans can also occur at lower temperatures (25-28°C) in in vivo systems such as zebrafish (Brothers et al., 2011) or C. elegans (Pukkila-Worley et al., 2009), and plays a crucial role in pathogenesis in these systems. This indicates that additional host-related factors or conditions can supersede the need for increased...
temperature in these models. Furthermore, the ability to grow at 37°C itself can be considered a virulence factor of its own in pathogenic fungi (Casadevall, 2005) and other microbes. For example, the calcineurin pathway is required both for full virulence, and for growth at temperatures of 37°C and above in *C. glabrata* (Chen et al., 2012) and *Cryptococcus neoformans* (Odom et al., 1997). To test these temperature-dependent aspects of pathogenesis, the suitability of many alternative infection models is limited, with few exceptions, such as *G. mellonella*, which withstands temperatures up to 37°C (Desalermos et al., 2012).

In addition, although the evolutionary ancient Toll pathway shares many similarities with its counterpart in mammalian innate immune recognition pathways (Ferrandon et al., 2007), the immune systems of humans and insects differ fundamentally in many aspects. Certain fungal immune evasion factors are, therefore, likely to escape detection in mutant screens using these models. Mammalian and other vertebrate hosts, such as zebrafish (Gratacap and Wheeler, 2014; Tobin et al., 2012), are better suited in this respect. In these models, several genes of related functional classes were found to be relevant in both models, although, interestingly, some seemed to be specific for each infection model.

**Implications and future directions**

This mutant library screen is one of the largest to date to compare a vertebrate and an invertebrate infection model. The data obtained are especially relevant for future large-scale mutant library screens of pathogenic microbes: a broad screen in an invertebrate host can pre-select conspicuous mutants to be validated and investigated in-depth in the ethically and practically more-challenging mouse models. This will allow researchers to better judge the applicability of data from alternative models, and help reduce and refine the use of mice in infection biology research.

**Results**

This work establishes a systemic infection model of mice for the simultaneous investigation of large sets of mutant strains of the human pathogenic fungus *Candida glabrata*, and an individual infection screen for hundreds of fungal mutants in fruit flies. When comparing these models, the relative fitness of mutants of *C. glabrata* in murine infections was predicted by using an alternative *Drosophila melanogaster* infection system. Moreover, fly mortality predicts growth of fungal mutant strains in mouse organs better than *in vitro* pre-screens, indicating similar infection-specific functions of many fungal genes in both models. Finally, several genes of related functional classes were found to be relevant in both models, although, interestingly, some seemed to be specific for each infection model.

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**Disease Models & Mechanisms**

**TRANSLATIONAL IMPACT**

**Clinical issue**

Understanding mechanisms of infectious diseases requires suitable animal models for replicating human infections *in vivo*. Mammals such as mice are generally the model of choice because they closely resemble humans in many – although not all – biologically relevant aspects, like general anatomy and immune functions. However, for ethical and practical reasons, alternative models, ranging from vertebrates (like zebrafish) to insects or even amoebae, are increasingly used. Obviously, these models deviate substantially from humans. Hence, the use of alternative infection models is accompanied by an ongoing debate about how well these models reflect disease in mammals and, ultimately, humans.

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**Disease Models & Mechanisms**

**RESULTS**

To compare the *Drosophila melanogaster* and *Mus musculus* microbial infection models, we used a recently created large-scale library of *C. glabrata* gene-deletion mutants, enriched for processes that are likely to be involved in pathogenesis or transcriptional...
The D. melanogaster model

We started by testing the ~400 C. glabrata mutants of the gene deletion collection by using our immunodeficient (Toll-signaling-defective; MyD88) D. melanogaster model (Quintin et al., 2013). The biologically relevant outcome in this model was the death of the host flies, which was recorded on a daily basis. We used the Weibull distribution originally suggested by Glittenberg et al. for C. albicans (Glittenberg et al., 2011) to fit the Kaplan–Meier survival plots obtained by these experiments (Fig. 1A). As a measure for virulence, we calculated a fly virulence index (FVI) of all mutants (supplementary material Table S1). This was done by determining the time (LT\textsubscript{50}) at which 50% of D. melanogaster had died according to the model. The log\textsubscript{2} ratios of these LT\textsubscript{50} values were calculated between flies infected with the deletion mutant and those infected with wild-type C. glabrata in the same infection group (see Materials and Methods for a more detailed description).

Most mutants did not deviate much from the wild type in their virulence when using this measure, as would be expected from a large collection of single-gene deletions. A Gaussian distribution fit for the FVI data has a mean of ~0.074, corresponding to a 5% increase in LT\textsubscript{50} when using the mutants compared with the wild type (Fig. 1B). We set the FVI cut-off for changes signifying an increased or decreased virulence in the fly to ±0.5 (1.4-fold), which we considered potentially biologically relevant. Altogether, 58 mutants (13.5%) were classified hypovirulent, and 23 mutants (5.4%) were hypervirulent. These comparatively high values were not unexpected, as the original mutant collection was already enriched for mutants with potential virulence defects (Schwarzmüller et al., 2014). Furthermore, nine of these mutants (2.1%) could be classified as strongly hypovirulent (FVI<1.0) and three (0.7%) as strongly hypervirulent (FVI>1.0).

We used previously published in vitro growth data of these mutants to expand our analysis. In this context, reduced growth was defined as a >2\(\sigma\) increase in generation time in complex medium compared with the mean of all strains (Schwarzmüller et al., 2014). The percentage of mutants with reduced in vitro growth was similar to the proportion of hypovirulent strains by FVI values (13.7% vs. 13.5%). Similarly, 6.5% of mutants showed increased (>2\(\sigma\)) growth in vitro, compared with 5.4% that were hypervirulent in flies. There is some correlation (r=0.43) of the fly virulence index to the relative growth rate of the mutants in vitro (Schwarzmüller et al., 2014), but many of the most strongly attenuated mutants in vivo still showed normal in vitro growth rates (see below).

A gene ontology (GO) slim-term enrichment analysis of the sets of mutants that were attenuated in Drosophila virulence revealed an over-representation of biological processes related to the response to stimuli and signal transduction – but also maintenance of homeostasis – in the hypovirulent mutants, even after taking the library-creation bias into account (Table 1). No significant GO term enrichment was found for the hypervirulent mutants.

Genes important for cell wall integrity scored among the most strongly attenuated in our Drosophila model (Table 2, supplementary material Table S1). Deletion of the homologs of SSD1 (with normal in vitro growth) and its regulator CBK1 (with reduced growth), representing the cell wall integrity part of the RAM network (Saputo et al., 2012), led to strongly hypovirulent mutants (FVI<1.0). Similarly, deletions of genes involved in the Stl2/Mpk1 MAPK cascade for cell wall biogenesis (ROM2, PKH1, PKH2, YPK2, MKK2 and RLM1) are all hypovirulent or strongly hypovirulent without any measurable defect in in vitro growth. Deleting the main chitin synthase gene CHS3 of the cell wall or a gene encoding a part of its Golgi-to-membrane exomere transport complex (CHS5) also led to hypovirulent C. glabrata mutants in our system. Finally, disturbing the glucan part of cell wall biogenesis also appeared to reduce virulence in flies: deletion mutants of the glucan synthase genes FKS2 and FKS3 both had FVI values of less

[Fig. 1. Fly virulence index (FVI) of 428 C. glabrata mutants. (A) Data fitting for FVI calculation is shown in two examples (CTA1- and MNN10-deletion mutants). The calculation was based on a Weibull distribution fit to the fly survival data. For each experimental group, the LT\textsubscript{50} ratio of mutant to wild type was calculated. Black circles, measured fly survival for mutant strain, gray triangles, corresponding wild-type data; solid graph lines, curves calculated by Weibull formula; vertical lines, LT\textsubscript{50} values. (B) Distribution of the FVI. In the mean, mutants were slightly attenuated in the fly, with a Gaussian fit mean of ~0.074. White and light gray, neutral mutants, gray, increased or decreased virulence, black, highly increased or decreased virulence. (C) Deletions of HOG pathway genes lead to reduced virulence in flies. All tested deletion mutants had FVI values of ~0.36 or less (see color scale; gray indicates no data). A similar reduction in virulence was obtained in mice (MVI) for deletion of SHO1 but not of SSK1. Note that the Sln1 arm of the pathway is disrupted due to a non-functional Ssk2 in C. glabrata ATCC 2001. Modified from Gregori et al. (2007).]
than −0.5 (fksΔ was not part of the mutant collection). None of these were defective in in vitro growth. MNN10, as a gene encoding a mannosyltransferase with function in barrier septum formation, was required for full fly virulence (mutant FVI of −0.54) but also for normal in vitro growth. Strains lacking the aspartic proteases Yps7 and Yps10 were also decreased in virulence in the fly model, an observation reminiscent of our recent finding that a ypsl-1Δ strain lacks virulence in the Drosophila model (Quintin et al., 2013). Yps7 has recently been associated with cell wall integrity (Bairwa et al., 2015). Surprisingly, the catalase deletion mutant cta1Δ was also hypervirulent in our model (Fig. 1A). Overall, cell wall integrity pathways seemed to be paramount for C. glabrata virulence during Drosophila infections, as were selected genes for growth and immune cell interaction.

### The murine pool model

We devised a mutant pool approach to test the C. glabrata strains. In our systemic mouse infection model, animals do not succumb to C. glabrata infection, which allows for long-term experiments to observe competition of mutants within a pool. Four pools were set up with approximately 40 mutants each (supplementary material Table S2), representing hyper- and hypovirulent mutants as well as mutants with normal virulence in the fly model. Assignment to each pool was random for all strains. In our systemic mouse infection model, animals do not succumb to C. glabrata, all animals remained clinically healthy, comparable to single-strain challenge experiments (Jacobsen et al., 2010a). The fungal burden in the organs at the three sampling days (Fig. 2A) were also in very good agreement with our previous single-strain data (Jacobsen et al., 2010a), indicating a normal progression of disease in the animals.

### Table 1. GO slim-term enrichment of genes deleted in mutants less virulent in flies (FVI−0.5)

| GO ID   | GO-term                                      | P-value | FDR  | Mutants |
|---------|----------------------------------------------|---------|------|---------|
| 0005716 | Cellular response to stimulus                | 0.0014  | 0.0033 | 17      |
| 0050794 | Regulation of cellular process               | 0.0014  | 0.0040 | 17      |
| 0044700 | Single organism signaling                    | 0.0014  | 0.0050 | 17      |
| 0023052 | Signaling                                    | 0.0008  | 0.0087 | 17      |
| 0007165 | Signal transduction                          | 0.0014  | 0.0100 | 17      |
| 0007154 | Cell communication                           | 0.0014  | 0.0200 | 17      |
| 0050896 | Response to stimulus                         | 0.0016  | 0.0029 | 32      |
| 0044267 | Cellular protein metabolic process           | 0.0050  | 0.0275 | 17      |
| 0099887 | Cellular process                             | 0.0059  | 0.0244 | 44      |
| 0019538 | Protein metabolic process                    | 0.0060  | 0.0220 | 18      |
| 0044763 | Single-organism cellular process             | 0.0070  | 0.0200 | 37      |
| 0043412 | Macromolecule modification                   | 0.128   | 0.5000 | 15      |
| 0065008 | Regulation of biological process             | 0.0141  | 0.0435 | 7       |
| 0042592 | Homeostatic process                          | 0.0141  | 0.0462 | 7       |
| 0019725 | Cellular homeostasis                         | 0.0141  | 0.0493 | 7       |

Signaling, protein metabolism and homeostasis mutants are enriched in these strains. Only terms with a false discovery rate (FDR) of <0.05 are shown.

### Table 2. The 20 C. glabrata mutants with the lowest indices for Drosophila (FVI, left) or mouse (MVI, right) infection

| Drosophila | FVI  | Systematic name | Name* |
|------------|------|-----------------|-------|
| #          |      |                 |       |
| 1          | −1.96| CAGL0J00672g    | CBK1  |
| 2          | −1.90| CAGL0L06138g    | TPN1  |
| 3          | −1.50| CAGL0H01287g    | SSD1  |
| 4          | −1.37| CAGL0H08437g    | VPS15 |
| 5          | −1.33| CAGL0I07513g    | PKH1  |
| 6          | −1.04| CAGL0F01507g    | SLG1  |
| 7          | −1.03| CAGL0M01628g    | SAC7  |
| 8          | −1.02| CAGL0G03597g    | SHO1  |
| 9          | −1.00| CAGL0G04873g    | ROM2  |
| 10         | −0.97| CAGL0L01331g    | ANP1  |
| 11         | −0.90| CAGL0B04389g    | CHS3  |
| 12         | −0.89| CAGL0K04037g    | FKS2  |
| 13         | −0.88| CAGL0H07403g    | KRE2  |
| 14         | −0.85| CAGL0L08910g    | AEP3  |
| 15         | −0.85| CAGL0I09130g    | PTR3  |
| 16         | −0.84| CAGL0E01353g    | ZRT2  |
| 17         | −0.83| CAGL0I08195g    | GPA2  |
| 18         | −0.83| CAGL0L06267g    | GPB1  |
| 19         | −0.80| CAGL0E02629g    | ALG6  |
| 20         | −0.80| CAGL0K01507g    | GPR1  |

| Mouse      | MVI  | Systematic name | Name* |
|------------|------|-----------------|-------|
| #          |      |                 |       |
| 1          | −6.58| CAGL0F08041g    | PFK1  |
| 2          | −5.48| CAGL0H08437g    | VPS15 |
| 3          | −4.60| CAGL0J06072g    | CBK1  |
| 4          | −4.24| CAGL0D01034g    | VIG9  |
| 5          | −4.16| CAGL0I09130g    | PTR3  |
| 6          | −4.02| CAGL0M12507g    | VHR1  |
| 7          | −4.01| CAGL0G02827g    | SLM1  |
| 8          | −2.70| CAGL0G02827g    | D1D4  |
| 9          | −2.65| CAGL0H06545g    | ATG32 |
| 10         | −2.61| CAGL0J1231g     | MNN10 |
| 11         | −2.21| CAGL0L00627g    | GBP1  |
| 12         | −2.17| CAGL0L01331g    | ANP1  |
| 13         | −2.16| CAGL0I02519g    | YMR253C|
| 14         | −2.15| CAGL0I08195g    | GPA2  |
| 15         | −2.01| CAGL0A02431g    | YPS7  |
| 16         | −1.96| CAGL0H01287g    | SSD1  |
| 17         | −1.83| CAGL0G06864g    | MPS3  |
| 18         | −1.82| CAGL0M05533g    | DUR1  |
| 19         | −1.72| CAGL0M1826g     | ECM33 |
| 20         | −1.71| CAGL0J04312g    | PEP1  |

*Common name or S. cerevisiae homolog.

Mutants found in both lists are indicated in bold. The complete list can be found in supplementary material Tables S1 and S3.
As a biological meaningful measure, we calculated the depletion or enrichment of mutants relative to wild type after re-isolation. This was done by dividing their respective re-isolation-to-infection ratios (the mouse virulence index, or MVI; see Materials and Methods for details). At any given time point in any organ, the mean virulence indices of the mutants were found to be slightly lower than those of the wild type, indicating – as expected and similar to the fly model – that, whereas most individual mutants did not deviate much in fitness from the wild type, on average, gene deletions result in at least slight growth defects in the murine system (Fig. 2B). Over time, this tendency increased in all organs, with the exception of the brain, where the distribution of mutants fluctuated strongly. The latter was probably due to the low re-isolation frequency in brain, as it favors random fluctuations and ‘founder effects’ over actual selection pressure.

Using microarray analysis, we were able to detect most of the barcodes of the injected strains in all infected organs (supplementary material Table S3). For our analysis, we have used combined DNA of pools isolated from the same organ of three individual mice at each time-point. In order to validate this detection approach, we compared array data of selected mutants with quantitative PCR (qPCR) data obtained from the same samples isolated from the individual mice. The overall correlation between the array-based MVI and the means of the qPCR data was good (Pearson $r=0.78$, Fig. 3A) and in range of similar comparisons using expression data (Morey et al., 2006), with the exception of the kidney (liver $r=0.96$, spleen $r=0.90$, brain $r=0.87$, kidney $r=0.44$).

To further study the inter-replicate variation, we investigated the variation of the barcode signals of selected mutants between individual mice. We focused on mutants representing examples for both well-correlating, and differing FVI and MVI values. As a measure of scatter among individual mice, we calculated the coefficient of variation (CV), i.e. the standard deviation of the mean from qPCR measurements of individual organs in percent. As shown in Fig. 3B, the CV generally increased over time, indicating an increasing effect of random or individual and specific processes on the relative frequency of the mutants. Similarly, the frequency of mutant re-isolation from the brain shows a very high inter-individual CV already at day 2 post infection (p.i.), again hinting at a dominance of random events over genetic determinants in the colonization of this organ by *C. glabrata*. At day 7, the organs with the smallest differences between individual mice were the liver and the spleen. Given all these observations and the low colony forming unit (cfu) counts from the brain, we decided to exclude the brain from our further analysis, and defined the total MVI as the mean value over all time points obtained from liver, spleen and kidney.

Based on the total MVI of individual genes, the most strongly depleted mutants were deletion strains of: *PFK1* – similar to the fly
model; a kinase involved in vacuolar protein sorting (VPS15); the RAM network kinase gene CBK1 – again similar to the fly model; PTR3, probably encoding part of the sensor complex for external amino acids; the pseudogene CAGL0M12507g, which is homologous to the S. cerevisiae vitamin H transporter transcriptional activator gene (VHRI); and SLM1, probably encoding a downstream effector of the TOR pathway. Furthermore, deletion of DID4, whose homolog is also involved in vacuolar protein sorting, the mitophagy-related gene ATG32, and the genes involved in protein mannosylation, MNN10 and VIG9, led to a strong reduction in overall fungal burden. Of these ten deletion mutants, five also had moderate (VPS15) to severe (PFK1, CBK1, VIG9, MNN10) in vitro growth defects, whereas – in contrast – one mutant (DID4) displayed an increased in vitro growth rate. Overall, there is only some correlation (r=0.31, P<0.01) of the MVI with the published in vitro fitness of the mutants (Schwarzmüller et al., 2014; supplementary material Fig. S1). Further attenuated mutants included, among others, yps5A (CAGL0E01771g) and yps7Δ, and – like in D. melanogaster – deletion mutants of genes involved in cell wall biosynthesis and maintenance (ECM33, CHS1), and protein glycosylation (ANPI).

Among the ten mutants enriched in the organs (mean MVI>0.5) were TIP41, an ortholog of a negative regulator gene of the TOR pathway (confirming the importance of the TOR pathway that was implied by attenuation of mutants such as slm1Δ), CKB2, encoding the inhibitory subunit of the protein kinase CK2 in S. cerevisiae, and GRP1, which in baker’s yeast is involved in glucose detection. The AXL2 and BNII genes, both involved in axial bud site selection, also showed increased MVIs. In contrast to the depleted mutants with low MVIs, however, these enrichments were largely represented by a single organ, the kidney.

**Correlation between the models**

We went on to analyze how far the data obtained from the fly model can predict the outcome of the murine model experiments. In a first step, we calculated the correlation coefficients of the FVI and the MVI of individual organs and time points (Fig. 4A-C). As can be seen in Fig. 4D, the degree of correlation depends both on the organ and the time post infection. Reasonable and statistically significant correlation was found between fly and mice data for day 7 p.i. in the target organs liver (r=0.47, P<0.001) and kidney (r=0.37, P<0.001) (Fig. 4A,B), whereas in general the brain organ burden showed poorer correlation with the fly survival rate. Earlier or later time points than day 7 generally, but not always, showed less correlation between the individual organ and the fly data. We found essentially no correlation between the fungal burden in the murine brain at day 2 p.i. and the fly survival time for our mutants (Fig. 4D).

We calculated the total MVI (the mean over all organs – excluding the brain – and all time points) for every mutant as a crude and very basic measure for total fitness of the mutant relative to the wild type over the whole course of our experimental infection. This MVI had a reasonable correlation with the fly survival times of r=0.42 (indicated as dashed line in Fig. 4D). Overall, the predictive value of fly survival time for the fungal burden of any given mutant is, therefore, best for the target organ liver at 7 days after infection.

As a final and biologically relevant measure, we wanted to investigate whether virulence data obtained from the fly model is able to predict the overall virulence phenotype in mice. To this end, we simplified the MVI to three classes: hypovirulent (MVI<−0.5), neutral (MVI −0.5 to 0.5) or hypervirulent (MVI>0.5). We employed a multinomial logistic regression model (see Material and Methods) to use the FVI as a predictor for the mouse virulence class of the mutants. With the classes defined by the total MVI, we obtained a good predictive value of the FVI for differentiating the hypovirulence and neutral class (P<0.01, Table 3 and Fig. 5A). The coefficient for the hypovirulence-to-neutral class transition was −1.07, i.e. an increase of the FVI by one unit decreased the log odds ratio of the mutant to be hypovirulent versus neutral in mice organs by 1.07 (Table 3). For example, an FVI of −2 in a mutant corresponds to a 86% probability of hypovirulence and 11% of neutral behavior in mice (Fig. 5A); at an FVI of −1, these probabilities are changed to 70% and 24%, respectively. Overall, a low FVI, therefore, predicts a high probability of any mutant to be depleted in mice organ colonization.

Interestingly, the neutral class becomes the most accurately predicted (>45%) only for mutants with an FVI of zero or more. In this case, an MVI virulence class on the basis of the liver data from day 7 p.i. provides better fitted results. Based on this comparison of liver data against FVI, changes in the FVI also predict well the difference between hypovirulent and neutral mutants (coefficient −1.35, P=0.001), but an FVI of more than −0.25 already predicts the neutral class in mice as most probable (Fig. 5B). As a control, the
same model was generated with brain data from mice 2 days p.i., which did not correlate well with the FVI. As expected, the coefficient was very low (−0.2) and not statistically significant (Table 3, Fig. 5C). Similarly, completely randomized FVI-MVI pairs held no measurable predictive value (data not shown). Overall, the fly virulence index had the highest predictive power for mouse liver burden at day 7 p.i.

Interestingly, using the *in vitro* growth as the basis, the prediction of the murine virulence class is not as reliable. For the total MVI (excluding the brain), a slow *in vitro* growth predicts a hypovirulent mutant with a maximum probability of 60%, and hypervirulence is estimated to have a 40% chance (Fig. 5D) – compared with 86% and 3% probability, respectively, with the FVI as a predictor. Only slightly better results were obtained for liver at day 7 post infection

Table 3. Coefficients (Coeff.) with standard deviations (s.d.) and *P* values of the multinomial logistic regression models for the prediction of MVI or FVI hypovirulent and hypervirulent classes

| Prediction | Hypovirulent class | Hypervirulent class |
|------------|--------------------|---------------------|
|            | Coeff. | s.d. | *P* | Coeff. | s.d. | *P* |
| Total MVI (except brain) by FVI | −1.066 | 0.381 | 0.005 | −0.093 | 0.608 | 0.878 |
| Liver 7 days p.i. by FVI | −1.349 | 0.410 | 0.001 | −0.194 | 0.536 | 0.717 |
| Brain 2 days p.i. by FVI | −0.209 | 0.487 | 0.667 | −0.618 | 0.478 | 0.196 |
| Total MVI by *in vitro* | −5.050 | 2.179 | 0.020 | −6.921 | 2.611 | 0.008 |
| Liver 7 days p.i. by *in vitro* | −2.739 | 1.679 | 0.103 | −2.774 | 2.167 | 0.201 |
| FVI by *in vitro* | −7.512 | 2.245 | 0.001 | 6.362 | 4.356 | 0.144 |

The neutral class (−0.5 < MVI/FVI < 0.5) served as reference class. Hypovirulence in mice can be sufficiently predicted from the FVI for classes based on the overall MVI (except brain; see Fig. 5A) and for the liver MVI 7 days p.i. (see Fig. 5B), whereas hypervirulence cannot. Prediction of fungal burden in the brain is impossible from fly data, as depicted here for day 2 p.i. (see Fig. 5C). Prediction of MVI (both total and for liver day 7) from *in vitro* data is much less reliable than from the FVI (see Fig. 5D, E), whereas the FVI is predicted well from *in vitro* growth (see Fig. 5F). For the coefficients, note the different ranges of FVI and *in vitro* growth indices as basis for the models.
severely. In contrast, deleting those genes had little (probability of any mutant to be hypervirulent in mice (total suited.

To predict murine virulence classes, the FVI is much better reliably predict the virulence class of a given mutant in flies but not least for hypovirulence in flies (Table 3). Hence, predicting hypovirulence; Fig. 5F) and statistically significant, at least for virulence classes, the FVI is much better suited. For all hypervirulent mutants, good prediction of murine virulence from increased growth of mutants is not immediately possible, although the differences between the models, with moderate to strong attenuation in mice, and an unchanged or increased virulence in flies. All these deletions led to little to no in vitro growth defects (supplementary material Table S3; vitro growth is not as good a predictor of the mouse virulence class as the FVI: compared with A and B, negative indices predict hypovirulence less reliably. (F) The fly virulence class is predicted well by the in vitro growth rate of C. glabrata. See also Table 3 for detailed data.

(Fig. 5E). Again, the prediction of virulence class in the mouse model by using the FVI outperforms the in vitro data considerably (see also Table 3). Strikingly, the prediction of the FVI class itself by the in vitro growth index is good (with an index <−0.5 reliably predicting hypovirulence; Fig. 5F) and statistically significant, at least for hypovirulence in flies (Table 3). Hence, in vitro growth can reliably predict the virulence class of a given mutant in flies but not mice. To predict murine virulence classes, the FVI is much better suited.

Individual differences of mutants between mice and flies
Overall, a decrease in FVI, therefore, is likely to indicate a defect in the murine infection model for the mutant. Looking at results from the mutants that showed large deviations between virulence indices in mouse and fly can give insight into differences between the models, as experienced by the fungus during the infection process. Here, for example, strong differences are evident in parts of the oxidative stress response. In flies, deletion of the genes encoding the main catalase (CTA1), the regulator of oxidative stress response (MSN2) or the copper transporter necessary for function of superoxide dismutases (CTR2) increases the FVI slightly to 0.5 reliably growth can reach a >40% probability of causing hypervirulence in flies (Fig. 5F).

CDC12) often – but not always – showed stronger defects in mice than in flies (supplementary material Table S3). Additionally, deletions of genes involved in later steps of outer chain protein N-glycosylation, such as MNS1, MNN4 or GNT1, also led to strong differences between the models, with moderate to strong attenuation in mice, and an unchanged or increased virulence in flies. All these deletions led to little to no in vitro growth defects (Schwarzmüller et al., 2014). The deletion mutant strains of MNN10, ANP1 and VIG9, involved in earlier synthesis steps of the mannan backbone for N-glycosylation, showed both reduced FVI and MVI.

Yet, two of these mutants, mnn10Δ and vig9Δ also had severe in vitro growth defects (supplementary material Table S3; Schwarzmüller et al., 2014), and the same may be assumed for anp1Δ as Anp1 forms a complex with Mnn10 in S. cerevisiae (Jungmann et al., 1999).

Finally, one arm of the HOG pathway is disrupted in C. glabrata ATCC 2001 (Gregori et al., 2007). In the remaining functional part of the HOG pathway, the deletion of both SHO1 and PBS2 led to reduction in fly and mice virulence (Fig. 1C). A deletion mutant of the Ssk1 kinase (which is part of the non-functional arm of the HOG-signaling pathway) has an FVI of ~0.51 (similar to the other HOG pathway mutants) but a neutral MVI of 0.04.

DISCUSSION
No infection model perfectly mimics human infection, and choosing the optimal model for the biological question at hand needs deliberate consideration of each model’s advantages and disadvantages. Reproducibility, ease of setup and evolutionary distance to humans, as well as cost-effectiveness and ethical considerations must be taken into account (Maccallum, 2012). In many cases, it is preferable to use a simple and accessible system for choosing the optimal model for the biological question at hand.

No infection model perfectly mimics human infection, and choosing the optimal model for the biological question at hand needs deliberate consideration of each model’s advantages and disadvantages. Reproducibility, ease of setup and evolutionary distance to humans, as well as cost-effectiveness and ethical considerations must be taken into account (Maccallum, 2012). In many cases, it is preferable to use a simple and accessible system for first a large-scale screening. A detailed investigation of the strains found in the first test can then be performed in the more complex infection model. However, in many cases it is unclear how far the invertebrate (or simple vertebrate) model predicts the strains’ behavior in a complex vertebrate model that is more similar to humans. Comparisons between models are often limited to a limited number of strains. Examples include the use of more than 30
Pseudomonas aeruginosa strains in G. mellonella (Jander et al., 2000), six Aspergillus fumigatus mutants (Chamilos et al., 2010) or 13 strains of C. albicans in D. melanogaster (Glittenberg et al., 2011), and 15 deletion mutants in embryonated chicken eggs (Jacobsen et al., 2011). Generally, a fair to good correlation was found between the simpler model and the respective murine model.

Our use of several hundred mutants in the fly, of which more than 100 were tested in mice, allows a good comparison of the D. melanogaster and M. musculus infection models. Similarly, a very recent study by Desalermos et al. screened 1201 C. neoformans deletion strains in a variation of invertebrate hosts, resulting in 12 strains that were further tested individually in mice (Desalermos et al., 2015).

**The fruit fly as a model host**

C. glabrata generally shows a lower virulence when compared with C. albicans, as observed in mice (Brieland et al., 2001; Jacobsen et al., 2010a) and in embryonated chicken eggs (Jacobsen et al., 2011). In agreement with this lower virulence across different model organisms, fully immunocompetent flies do not succumb to C. glabrata infection (Quintin et al., 2013). Hence, we used the Toll-pathway-deficient (MyD88) Drosophila model.

Virulence of C. glabrata mutants with strongly reduced in vitro growth was often decreased in flies, as compared with the wild type. Accordingly, our model can predict the virulence class of the fly from in vitro growth with a good degree of confidence. However, some mutant strains with reduced FVI did not show any in vitro defects. Deletion of SSD1 or that of the kinase gene PKH1 had no effect on C. glabrata growth in liquid medium (Schwarzmüller et al., 2014) but reduced virulence in flies severely. Hence, in the immunodeficient fly model, growth in complex medium was a good indicator, but not sufficient to fully predict disease and death in flies. Most probably, certain virulence-associated genes are specific for fitness in the host and have little or no function in vitro. Moreover, temperature-related phenotypes may be impossible to detect when using flies. A possible example are calcineurin pathway genes that are involved in thermotolerance (Chen et al., 2012): C. glabrata deletion mutants of CNB1, RCN1 and CRZ1 were only slightly reduced in fly virulence and, hence, were not included in the murine pools. However, CNB1 and CRZ1 are known to be required for full virulence of C. glabrata in mice (whereas a rcn1Δ C. glabrata strain does not have a discernible virulence defect) (Chen et al., 2012).

Our data show the importance of an intact cell wall for the C. glabrata infection process in D. melanogaster. Many genes involved in the maintenance of cell wall integrity, either through signaling or biosynthesis of cell wall components, yielded mutated strains with reduced virulence in Drosophila (Ben-Ami et al., 2011). As FKS1 of C. glabrata, but not of C. albicans, has been described as being functionally redundant with FKS2 in vitro (Katiyar et al., 2012), the reduced virulence of C. glabrata FKS2 in Drosophila was surprising. This hints toward a possible non-redundant role of FKS1 and FKS2 (and FKS3) of C. glabrata in vivo.

Interestingly, the regulator of cell wall integrity Cas5 was found to be important in both fruit flies and a murine model of C. albicans infections (Chamilos et al., 2009). In fact, Cas5 was the only of 34 tested transcription factors found to be important in Toll-mutant Drosophila. It is also important for C. elegans infections (Pukkila-Worley et al., 2009). Although we did not test the C. glabrata ortholog of Cas5, the data indicate a similar central role of a fully intact cell wall in C. glabrata during infections of fruit flies. It is possible that the absence of a functional Sln1-Ssk2 arm of the HOG pathway in the ATCC 2001 strain exacerbated defects due to deletions of cell-wall-related genes: the SHO1 arm of the HOG pathway has been implicated in cell wall integrity in C. albicans (Roman et al., 2005), and a deletion of SHO1 renders C. glabrata hypersensitive to cell wall stress (Schwarzmüller et al., 2014). If this were the case, it would further underline the relevance of cell wall integrity in Drosophila infections, especially in comparison to mice.

**The murine pool infection model**

Testing mutants from large-scale deletion libraries as pools of dozens of strains has been successful for several fungal pathogens in the past. Pools of ten C. albicans transcription factor mutants were tested, for example, by Vandeputte et al. (2011), pools of 48 C. albicans mutants by Noble et al. (2010), and pools of 48 Cryptococcus neoformans mutants each by Liu et al. for 1201 strains in total (Liu et al., 2008). Here, we have successfully used a pooled approach with microarray-based detection for a murine C. glabrata infection model. We validated our system by using qPCR of selected mutants and found a very good agreement, with – for unknown reason – the exception of the kidneys. Overall, however, microarray data from the kidney agreed well with spleen and liver (supplementary material Table S3), suggesting a similar behavior of most mutants in these three organs. Remaining differences can be explained by the specific conditions encountered in the organs, ranging from overall geometry, nutrient supply and presence of organ-specific host molecules to differences in immune cell populations and cytokine profiles. The latter is known to play an important role, for example, in establishing C. albicans infection of the kidneys (Lionakis et al., 2013). As expected by the low re-isolation counts, the brain data deviated strongly from those of the other organs. Hence, we calculated the MVI by combining data only from liver, spleen and kidney.

Our results show that the overall disease progression of pool-infected mice was not different from established single-strain infection models (Jacobsen et al., 2010a). The pool size of approximately 40 strains was chosen to keep the total number of mice in the experiment low, while enabling us to detect larger changes in mutant abundance. Smaller pool sizes would likely provide a better resolution for measuring depletion or enrichment of individual mutants, and alleviate some of the problems caused by presumed population bottlenecks in the brain. However, a reduction in pool size would also increase the number of mice needed per experiment, and fluctuations due to population bottleneck remain a problem even with pools consisting only of a few strains (Jacobsen et al., 2010a). With 40 strains per pool, we were able to detect the vast majority of mutants using microarrays. As expected, the mutants were generally depleted compared with the wild type. The most strongly depleted strains were often, but not always, mutants with a strongly reduced in vitro growth (Schwarzmüller et al., 2014).

On the one hand, this validates our system, as we expected deletion mutants of genes such as PFK1 to be severely reduced in growth in vivo. On the other hand, even mutants strongly deficient in in vitro proliferation, such as ckb2Δ, were not necessarily reduced in virulence, and many mutant strains with low fly and mice virulence were unchanged in their in vitro growth (supplementary material Fig. S2). Finally, mutant strains with known defects in murine...
Correlation between mice and flies

The problems in comparing the fruit fly model – in which death of the host is the read-out parameter, with the murine model – in which fitness and growth of the pathogen serves as the measure, are self-evident. For these and other reasons, the usefulness of invertebrate models is often hotly debated. Yet, for each of these infection models, the above are the biologically relevant and – equally important – measurable parameters. Furthermore, although in our model mice do not succumb to C. glabrata infection, a strong correlation between fungal organ burden and virulence (measured by survival time) has been shown, e.g. for C. neoformans mutants (Liu et al., 2008). To enable a comparison between these two models, we have introduced the virulence scores. In mouse, an increase or decrease by one MV1 unit correlates to a twofold increase or decrease in the relative in vivo growth of the mutant compared with wild type. In fly, a change by one on our FVI scale equals a twofold shorter or longer mean survival of the host after infection with a mutant strain, again compared with wild type. We think this represents the best combination of a biologically relevant outcome and an experimentally accessible readout for each model. Alternative approaches, such as the fungal burden in immunocompetent D. melanogaster, are much more difficult to quantify and, in this case, would not be as good a virulence indicator as fly death. In contrast, monitoring mouse mortality, for instance, by using specialized immunocompromised models (e.g. Atanasova et al., 2013; Ju et al., 2002), cannot be used for pool experiments as the death of the host would negate any competitive advantages due to relative fitness of the strains. For these reasons, we employed the two models and indices described above. We set the cut-offs for hypovirulent mutants in both models to ±0.5, equivalent to a 1.4-fold difference compared with wild type. Other, e.g. more stringent, cut-off values are feasible, depending on the type of analysis planned. In our experiment, we considered 0.5 a good cut-off that is likely to signify biological significance.

The absolute values of the correlation coefficient in our experiments differed strongly depending on the murine organ under investigation. However, given the limitations in comparing these highly different systems, the correlation between our two models strikes us as remarkably informative. Interestingly, the correlation between fly and mice virulence scores is better than between in vitro growth (Schwarzmüller et al., 2014) and the relative mice organ burden, which is in agreement with the multinomial logistic regression model. It is possible that different, more host-related in vitro growth conditions (such as nutrient-poor or less oxygenated conditions) will result in a better agreement with in vivo fitness. However, the changing conditions and defences encountered in the host are unlikely to be simulated within any in vitro system, whereas the fruit fly model presents many of the stresses encountered in mammals.

In summary, we were able to establish a pool infection model for C. glabrata mutants in mice, and to show that our data are generally in good agreement with previous studies that used single mutants. Remaining differences may be due to inherent limitations of pool experiments, like the possible in trans complementation of virulence defects through co-infecting strains. This can be resolved in future experiments by using mutants of interest for single-strain infections. In our model, the liver showed a high fungal burden and a consistent decrease of the burden over time, and a low inter-individual variation at early and mid-time-points, making it the most robust target organ for barcode-based in vivo detection of C. glabrata pools. This model, therefore, seems highly suitable for future applications in competitive screening of C. glabrata mutants.

Specific differences and similarities

The differences observed between the models for some of the tested mutants may be informative with respect to the hosts’ differing responses. On the one hand, several aspects of immunity, such as the adaptive response and certain kinds of innate immune cell, e.g. dendritic and natural killer cells, do not exist in Drosophila. The melanization reaction of Drosophila, on the other hand, has no counterpart in mammals, although it cannot be activated in Toll pathway mutants (Ligoxygakis et al., 2002). Certain aspects of interaction between the fungus and the immune system might, therefore, differ substantially between the models, especially when – necessarily – using Toll-cascade-deficient flies.

So far, for example, it is unknown whether Drosophila hemocytes use an oxidative burst to kill ingested fungi. Whereas C. glabrata is known to be phagocytosed by hemocytes (Quintin et al., 2013), we found that a strain harboring a deletion of the single catalase gene CTA1 in C. glabrata (Cuellar-Cruz et al., 2008) kills the flies even faster than the wild type. The same is true for Msn2, a transcription factor involved in the general stress response including oxidative stress (Cuellar-Cruz et al., 2008), and Ctrl1, a Cu2+ transporter required for full superoxide dismutase activity. This suggests that these antioxidative activities are either highly redundant (Briones-Martin-Del-Campo et al., 2014) or not required for survival in our Drosophila model in vivo. Previously (Quintin
et al., 2013), we have asked whether the C. glabrata catalase plays a role in withstanding the cellular immune response in Drosophila. The data presented here at least indicate that this is not the case because phagocytosis of C. glabrata by hemoocytes takes place in our Drosophila model (Quintin et al., 2013), but the oxidative stress response seems dispensable for fungal virulence during fly infections. For the catalase Cta1, our findings confirm previous murine systemic infection experiments with C. glabrata where Cta1 also did not play a role (Cuellar-Cruz et al., 2008, and shown here). In contrast, however, our data indicate an important role of the transcriptional regulator Msn2 in murine infections.

An interesting aspect is the role of the HOG pathway in infections. C. glabrata ATCC 2001 is lacking one of the two branches of this osmotic stress resistance system and, therefore, relies solely on Sho1-Pbs2 signaling for osmoprotection (Gregori et al., 2007). Hence, the HOG pathway is completely disrupted in the sho1Δ and the pbs2Δ mutant, and both were found to be decreased in fly virulence. Whereas the pbs2A C. glabrata strain was not tested in the mouse model, we found the sho1Δ strain to be also strongly depleted in mice. In addition, we recently found sho1A C. glabrata to be more susceptible to killing by human macrophages (Seider et al., 2014). This indicates that the sole remaining HOG pathway is necessary for full fly and mice virulence in C. glabrata, and is most likely to be relevant in (human) phagocyte-pathogen interactions. The HOG pathway is also of importance in C. albicans infections (Alonso-Monge et al., 1999), where Sho1 plays a role in sensing oxidative rather than osmotic stress. The latter function is not present in C. glabrata Sho1 (Gregori et al., 2007), which fits well with our observation that the catalase Cta1 was not required for full virulence. Finally, the Ssk1 kinase of the disrupted Sln1-dependent arm of the HOG pathway seems to still play a role in Drosophila, but not in murine infections with C. glabrata ATCC 2001.

Interestingly, many glycosylation-deficient mutants appeared either in both our systems or, specifically in mice only, as reduced in virulence. Early steps in N-glycosylation seem to have a huge effect in interaction with both hosts, showing the importance of glycosylation per se. Differences between the hosts appear with mutants of later steps in glycosylation and are, thus, likely to be due to different interactions between specifically glycosylated fungal proteins and their respective host receptors. The och1Δ mutant of C. albicans, defective in the initiation of the outer chain N-glycosylation, was described as hypovirulent in mice (Bates et al., 2006), but the homolog was not part of our tests. Interestingly, one of the strongly reduced C. glabrata mutants in mice (and to a certain extent flies) – mnn10A – has recently been found to be important for survival in macrophages and has been implicated in active alkalization of the phagosome (Kasper et al., 2014).

Conclusions

Animal experiments should always be guided by the principles of refinement, reduction and replacement (3R). We have defined two new measures for virulence in flies and mice, and established the corresponding methods to determine them. In mice, we were able to demonstrate the feasibility of using pools of up to 40 barcoded C. glabrata mutants to determine fitness and virulence potential in murine infections. We have also shown that, for C. glabrata and probably other pathogenic microbes, fruit flies are a suitable model to predict the outcome of murine infections, especially following infection with severely attenuated mutants. Using our approach, a pre-screening of mutants in the invertebrate model Drosophila provides a good estimate of the probability to find a reduced microbial burden in mouse host with the same mutant. This pre-screen can be especially useful for selecting mutants from large, systematic genome-wide collections of deletion strains for validation in the mouse model, using a minimum number of mice. Additionally, this system is of potential interest for microbial drug target screens. Drosophila infections were superior to in vitro growth assays in predicting reduced fitness of mutants in mice. The corresponding gene products, therefore, include putative drug targets that would not have been selected by in vitro growth assays alone but play important roles within the host. This seems especially relevant in the light of previous landmark papers on large-scale, pool-based screens of C. albicans and C. neoformans mutant libraries in mice (Liu et al., 2008; Noble et al., 2010). In these studies, there was a good general agreement between in vitro growth defects of mutants and decreased presence in target organs. The authors also found many mutants whose presence was depleted in organs despite their wild-type-like in vitro growth; screening of an alternative infection model may have detected many of these strains. Desalermos et al. recently added an interesting twist to this approach by suggesting to use a range of different invertebrate infection models to best cover different mechanisms of virulence (Desalermos et al., 2015).

The remaining differences between the fruit fly and murine models must be viewed in light of the system used. Both systems have their specific limitations and do not fully reflect infection in humans; and no decision can easily be made as to which mutant phenotype is ‘more real’ or ‘better’ than the other when the two models give different results. Even the canonical murine models do not fully reflect human infections: for example, mice have a different commensal microbiome, and typical risk factors in humans include compromised immunity, underlying diseases and old age, which are not represented in the murine model. With these limitations in mind, functional insight into the infection process can be gained especially by comparing the data from the models. Clearly, using the ‘simpler’ model for a preliminary screen is preferable for ethical, financial and practical reasons. These first models are not limited to Drosophila but can include, for example, C. elegans (Breger et al., 2007), G. mellonella (Borghi et al., 2014; Jacobsen, 2014), zebrafish (Gratacap and Wheeler, 2014) or embryonated eggs (Jacobsen et al., 2011). It is especially noteworthy that these simple models allow better predictions of murine organ burden than an in vitro growth assay. Virulence factors common to the simple and the complex model that, nonetheless, have no discernible in vitro phenotype can, thus, be detected more reliably.

MATERIALS AND METHODS

Strains and growth conditions

The deletion mutants are described elsewhere (Schwarzmüller et al., 2014). Briefly, all mutants are based on a triple-auxotrophic (trp1Δ, leu2Δ, his3Δ) derivative of the C. glabrata ATCC 2001 reference strain. Genes were replaced by a NAT1 marker cassette with one of 96 different genetic barcodes (Schwarzmüller et al., 2014). The reference wild type contains its own specific barcode. All strains were routinely grown in YPD complex medium at 30°C, 180 rpm.

D. melanogaster infection model

D. melanogaster survival experiments were performed as previously described (Quintin et al., 2013). Briefly, batches of 20-25 MyD88 mutant flies (Tauszig-Delamasure et al., 2002) were challenged by septic injury using a needle dipped into a concentrated solution of C. glabrata, or using a thin capillary filled with yeast cells resuspended in PBS (OD_{600}=20) containing 0.01% Tween to avoid agglutination. The thorax was injected with 9.2 nl using a Nanject II apparatus (Drummond Scientific, Broomall, Pennsylvania).
PA). Vials containing infected flies were put in an incubator at 29°C and surviving flies counted every day. Flies were moved into fresh vials every other day.

To calculate the fly virulence index (FVI), data on fly death rates were fitted to Weibull distributions as suggested for C. albicans (Gittenberg et al., 2011), and the time at which 50% of the animals had succumbed to the infection was calculated (LT50). For each infection group, a reference wild type was included. The FVI is the log2 of the LT50 ratio of mutant and corresponding wild type.

**Pool composition and murine infections**

On the basis of the D. melanogaster data, four randomized pools were composed comprising strongly attenuated, moderately attenuated, hypervirulent, and neutral control strains, plus the barcoded wild type (supplementary material Table S2). Strains were precultured individually overnight and mixed in equal amounts by their OD600. The pooled yeasts were washed twice with PBS and adjusted to 2.5×10^8 cfu/ml for infection.

Specific-pathogen-free, outbred, female, 5-week-old CD-1 mice weighing 18-22 g (Charles River, Germany) were housed in groups of five in individually ventilated cages and cared for according to the principles outlined in the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes. All animal experiments were in compliance with the German animal protection law and approved by the responsible Federal State authority and ethics committee (permit no. 03-008/07). For infection experiments, mice were intravenously challenged with 5×10^7 cfu in 200 µl PBS, and their health status was subsequently examined twice daily by a veterinarian. On days 2, 7 and 14 post infection, three animals per tested pool were sacrificed (Jacobsen et al., 2010a). Post mortem analysis included recording of macroscopical changes and determination of fungal burden. Kidneys, spleen, liver and brain were removed aseptically at necropsy, rinsed with sterile PBS, weighed and aseptically homogenized in PBS using an IKA T10 basic Ultra-Turrax (Ika, Staufen, Germany).

As the number of fungi inside organs was too small for direct isolation of DNA, homogenized organs and the original infection pool were plated and grown for 28 h. Plates with a maximum of 2000 colonies were used for DNA extraction after scraping in PBS (Sambrook and Russell, 2001). For nearly all organs and time points, sufficient colonies (i.e. hundreds) grew, except for in the brain at later time points. Data from these few colonies were initially included in the analyses, with the caveat that they might be unreliable due to the low sampling numbers.

**Array design and barcode detection**

Arrays were designed to detect the barcodes. Isolated fungal DNA was mixed with DNA standards containing additional barcodes. Barcodes were PCR-amplified and labeled with cyanine dyes. DNA isolated from the plated inoculation pools and from the re-isolated strains (pooled from three mice each) were labeled with different cyanine dyes for a two-color hybridization with dye swaps to obtain two technical replicates. Array data were evaluated using GeneSpring GX, Version 12.1 (Agilent, Santa Clara, CA). Only data with signal-to-noise ratios of >2 in the inoculum were included. The relative amount of a mutant following infection was determined by dividing the signal intensities of the mutant’s barcode in the inoculum by the signal intensity in the re-isolated pool. Enrichment or depletion of the mutant was calculated by dividing this ratio by the ratio obtained for the wild type for the same sample. A relative fito fitness of the mutant. The MVI is the log2 transformation of that ratio (Sig = signal intensity): MVI = log2[(Sig in inoculum/Mutant) / (Sig in inoculum/WT)]. Only mutant strains with at least six data points (half of the total of 12 organ/time pairings) were considered for further analysis.

**Statistical analyses**

GO term enrichment was calculated using GO::TermFinder (Boyle et al., 2004), with all mutants screened in the fly as background population. Sets of genes were considered enriched if P<0.05 and the false discovery rate Q<5%. GO terms were obtained from the Candida Genome Database (www.candidagenome.org). For correlation analyses, GraphPad Prism, version 6.00 (GraphPad Inc., La Jolla, CA) was used. For correlation analysis of organ means, mouse data were only used when values for >50% of organs and time points were available. Correlation values are given as Pearson correlation coefficient r.

For the multinomial logistic regression models, MVI and FVI were transformed into three categorical variables (virulence class), with the reference class neutral (−0.5<MVI/FVI<0.5), hypovirulent (MVI/FVI<−0.5) and hypervirulent (MVI/FVI>0.5). Predictor variables for the mouse virulence class were FVI values or previously published in vitro data – the relative growth in YPD medium at 30°C, compared with the average growth rate of the whole collection (Schwarzmüller et al., 2014). For fly virulence class, log2 converted in vitro data served as predictor variables. Models were fitted and coefficients were obtained by using R software (version 2.15.2) and the nnet package (version 7.3-5).

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**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

Experiments were designed and performed by S.B., J.Q., L.K., I.D.J. and E.H. Raw data were collected and evaluated by S.B., J.Q., L.K., M.E.R., E.H., T.S., C.d’E., D.F. and D.F. The models were created by S.B., L.K. and M.E.R. Data were interpreted by S.B., J.Q., L.K., I.D.J., M.E.R., C.d’E., K.K., S.R., B.H. and D.F. The manuscript was written by S.B., J.Q., L.K., M.E.R., B.H. and D.F. All authors were involved in editing and approving the manuscript for submission.

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**Supplementary material**

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