Specific Aims

Candida albicans is a commensal unicellular fungus known to cause oropharyngeal thrush and vulvovaginal candidiasis. More seriously is the life-threatening disseminated candidiasis with a 40% mortality rate that occurs in immunocompromised patients and is widespread in hospitals. Because *C. albicans* is becoming increasingly resistant to the few available antifungals, it is critical that we better understand this pathogen.

C. albicans interacts with the innate immune system in complex and dynamic ways. After phagocytosis by macrophages, this fungus can neutralize the pH of the phagolysosome and form hyphae to escape the and cause infection. Despite these fungi-macrophage interactions being critical for pathogenesis, *little is known about the pathways* that allow *C. albicans* to rapidly adapt to the harsh environment of the phagolysosome. Our long-term goal is to better understand these adaptations that *C. albicans* undergoes to survive within macrophages and the mammalian host.

An increasing amount of evidence suggests that alternative carbon metabolism plays an important role in pathogenesis. Our lab has extensively described the importance of amino acid catabolism and its role in fungi-macrophage interactions. Upon import and catabolism of amino acids, *C. albicans* releases ammonia, resulting in pH neutralization of the phagolysosome and triggering hyphal morphogenesis. Mutants defective in amino acid metabolism do not form hyphae inside macrophages and are significantly attenuated in both murine macrophage and disseminated candidiasis mouse models, highlighting the importance of alternative carbon metabolism.

Although amino acids are important for macrophage interactions, transcriptome data reveal that *other carbon sources, such as carboxylic acids, may also play an important role in this process*. My central hypothesis is that alternative carbon sources found within host niches serve as distinct signals to induce physiological changes to promote virulence and improve survival within the host. This hypothesis is supported by our own preliminary data. Deletion of the enzymatic machinery needed to use any one of these nutrients (amino acids, carboxylic acids, *N*-acetylglucosamine) attenuates virulence in the macrophage model. Additionally, *C. albicans* neutralizes the pH *without the release of ammonia* upon growth on carboxylic acids, indicating that each carbon source may elicit distinct responses. We will address our hypothesis by completing the following aims:

Aim 1 – Determine how carboxylic acids impact host-pathogen interactions

Eliminating *C. albicans'* ability to import carboxylic acids reduces its ability to neutralize the pH and decreases fitness when challenged with macrophages. However, the mechanism by which carboxylic acids promote virulence remains unclear. Our working hypothesis is that the sensing and catabolism of carboxylic acids allows *C. albicans* to modify the phagolysosome, improving fungal survival. Mutants defective in carboxylic acid metabolism will be characterized *in vitro* before studying *ex vivo* using our murine macrophage model and *in vivo* in the disseminated candidiasis mouse model to evaluate overall fitness.

Aim 2 – Determine the physiological adaptations upon exposure to host-relevant alternative carbon sources

Previous studies show that growth on lactate, a monocarboxylic acid, induces physiological changes that allow *C. albicans* to better survive within the host. We hypothesize that other host-relevant carbon sources, such as α-ketoglutarate, amino acids, and *N*-acetylglucosamine will yield similar adaptations that improve survival and enhance virulence. We will measure the physiological changes upon exposure to alternative carbon sources, including cell wall alterations, host cytokine production, and stress response. Biochemical assays and electron microscopy will be used to examine changes in cell wall. Stress response pathways will be tracked at the transcriptional and translational level.

Aim 3 – Elucidate the impact of abolishing alternative carbon metabolism

Elimination of amino acid, carboxylic acid, and *N*-acetylglucosamine metabolism independently results in a significant attenuation of virulence. We postulate the elimination of multiple carbon pathways will have an additive effect, significantly reducing *C. albicans'* virulence by removing its ability to effectively adapt to the mammalian host. The well-established CRISPR/Cas9 system optimized for *C. albicans* will be employed to generate a set of combinatorial mutants. Characterization of these mutants will first be done *in vitro* before utilizing macrophage and mouse models to assess virulence.

Successful completion of these aims will provide us with critical insight about *C. albicans'* virulence and its interactions with the host. This may result in improved treatment methods or novel drug targets.

Significance

Candida albicans is one of the most medically important fungal pathogens in humans. Although it commonly causes mucosal infections such as oral thrush and vaginal candidiasis, disseminated candidiasis, which occurs in hospitalized and immunocompromised patients, is the focus of most research[1]. With a mortality rate of 40% and steadily increasing infection rates, it is critical to understand *C. albicans* pathogenesis[2–4]. This yeast resides as a commensal organism in the oral cavity, and gastrointestinal and urogenital tracts[5]. However, disruption of the microbiota, use of medical implants, or a break in the gastrointestinal barrier can trigger *C. albicans* infections. Typically, the innate immune system and the antimicrobial activity of phagocytes appropriately clears pathogens, but *C. albicans* is able survive within a macrophage phagosome. Our lab is focused on understanding the intimate host-pathogen interactions that occur between macrophages and *C. albicans.*

The macrophage phagosome is a harsh environment designed to expose pathogens to a variety of antimicrobial agents, including reactive oxygen species (ROS), hydrolases, and an acidified environment. *C. albicans* has evolved to counter these antimicrobial agents and disrupt the acidification of the phagosome, allowing the pathogen to form hyphal projections and induce the cell death pathway in macrophages known as pyroptosis[6,7]. This results in escape from the macrophage and infection progression. Several transcriptional profiling experiments reveal that *C. albicans* shifts its metabolism upon phagocytosis to utilize alternative carbon sources such as amino acids, *N*-acetylglucosamine (GlcNAc), and carboxylic acids[8–10]. Our lab has shown that the shift in metabolism is directly connected to pathogenesis. For example, growth on amino acids or GlcNAc promotes pH neutralization and hyphal morphogenesis, requirements to survive in and subsequently escape the phagosome[7,11,12]. Utilization of host-relevant carboxylic acids, such as α-ketoglutarate (αkg) and lactate, are also important for pathogenesis, but in a manner that is distinct from the amino acid and GlcNAc pathways[13,14]. Growth on carboxylic acids does not induce hyphal morphogenesis and neutralizes the pH via a pathway independent of the GlcNAc and amino acid pathways, suggesting that *C. albicans* has carbon source-specific responses for survival within macrophages and the host. Regardless of the mechanism, each individual alternative carbon pathway is important for pathogenesis; deletion of the enzymatic machinery required to use any one carbon source attenuates virulence in the established murine macrophage model. Based on this evidence, I hypothesize that alternative carbon sources found within host niches serve as distinct signals to induce physiological changes, thereby promoting virulence and improving survival within the host.

I will assess this hypothesis of alternative carbon utilization and the subsequent physiological effects through a variety of *in vitro* and *in vivo* techniques. Aim 1 focuses on elucidating the details of the poorly-understood carboxylic acid-driven mechanism while Aims 2 and 3 more broadly focus on the role of several host-relevant alternative carbon sources. Successful completion of these aims will result in a greater understanding of *C. albicans* survival within phagocytes and the host, which is essential for identifying novel targets for antifungals and improving treatment methods.

Innovation

The aims proposed will address pending questions and yield novel findings regarding *C. albicans* virulence regulation. The elucidation of the carboxylic acid-driven pH neutralization mechanism has the potential to reveal promising antifungal targets. Understanding the physical adaptations *C. albicans* undergoes in the presence of alternative carbon sources is host-relevant and essential for developing treatment methods moving forward.

From a technical perspective, several experiments proposed require cutting-edge technology, several of which will be utilized in novel approaches. The CRISPR/Cas9 gene editing system has recently revolutionized the *Candida* field by exponentially expediting and simplifying the ability to edit the genome of this diploid organism[15]. I will take full advantage of CRISPR to generate the necessary mutants in a fraction of the time compared to the traditional method. Additionally, the pHluorin constructs proposed in Aims 1 and 2 will be utilized in a macrophage model for the first time, potentially resulting in exciting findings about the internal changes that occur within a *C. albicans* cell upon phagocytosis.

Aim 1: Determine how carboxylic acids impact host-pathogen interactions

C. albicans dramatically alters its expression profile upon macrophage phagocytosis. Amongst the most upregulated genes are those encoding for glyoxylate enzymes and the carboxylic acid transporters Jen1 and Jen2, suggesting that utilization of carbon sources such as lactate, pyruvate, and αkg are critical for pathogenesis. As shown in Figure 1, Jen1 and Jen2 are required for growth on mono- and dicarboxylic acids, respectively. Interestingly, Jen1 is mainly dispensable for pH neutralization in the presence of monocarboxylic

acids, which indicates a sensing mechanism is involved. Carboxylic acid import is essential for fungal survival and macrophage killing, implicating carboxylic acid utilization as a mediator of pathogenesis (Fig. 1B). Notably, the pH neutralization defects observed are independent from amino acid and GlcNAc utilization pathways[13].

My working hypothesis is the sensing and metabolism of carboxylic acids allows *C. albicans* to modify the macrophage phagosome by raising extracellular pH in a manner distinct from the previously described amino acid and GlcNAc mechanisms[7,12]. The objective of this aim is to understand the connection between carboxylic acid utilization and pathogenesis by understanding the pH neutralization mechanism, determining the importance of this pathway *in vivo*, and investigating additional components contributing to this process.

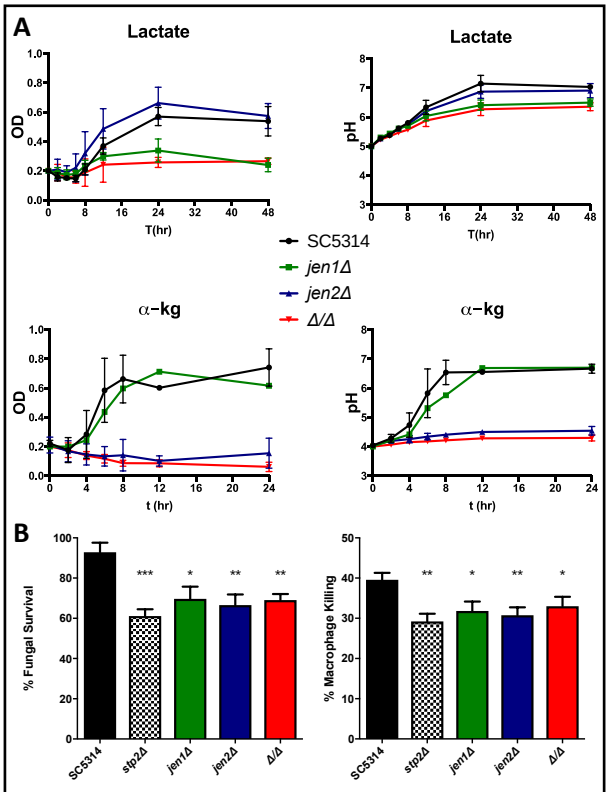

Figure 1 **Carboxylic acid importers are essential for pH neutralization and macrophage interactions. A)** The Jen1 transporter is required for growth, but not pH neutralization, in minimal medium containing monocarboxylic acids such as lactate. Jen2 is essential for both growth and pH neutralization on dicarboxylic acids such as αkg. **B)** Mutants incapable of importing carboxylic acids display significant decreases in fungal survival and killing of RAW 264.7 murine macrophages compared to SC5314(WT). *Δ/Δ* denotes *jen1Δ/jen2Δ*; *stp2Δ* is a negative control; required for amino acid utilization.(* p<0.05, ** p<0.001)

Subaim 1a. Understand the carboxylic acid-driven pathogenesis mechanism

The pH increase in the presence of monocarboxylic acids is independent from import, indicating that *C. albicans* can sense these molecules (Fig. 1A). This is consistent with recent reports of the lactate sensor Gpr1[14]. This G-protein coupled receptor initiates a signal for cell wall remodeling in the presence of monocarboxylic acids lactate and pyruvate, a process that is independent of import and metabolism. *In vitro* growth and pH assays of deletion mutants lacking Gpr1, its Gα subunit Gpa2, and/or the downstream transcription factor Crz1 will determine if the sensor and its associated proteins are responsible for pH neutralization in the presence of the monocarboxylic acids lactate and pyruvate. Additionally, pH assays with mutants defective for the glyoxylate cycle (*icl1Δ, icl1Δgpr1Δ*) or monocarboxylic acid import (*jen1Δ, jen1Δgpr1Δ*) will determine if metabolism and import contribute to pH neutralization independently of sensing[16]. Manipulating the extracellular pH may also alter pH homeostasis of *C. albicans* cytosol and vacuoles. Utilizing the newly-described pH-sensitive GFP construct “pHluorin” expressed in WT and *jen1Δ* will track the intracellular pH changes in the cytosol and vacuoles in the presence of mono- and dicarboxylic acids [17]. Contrary to monocarboxylic acids, the import of dicarboxylic acids is required for pH neutralization to occur (Fig. 1A). pH assays with the *icl1Δ* mutant will determine whether metabolism or import of dicarboxylic acids is the critical step[16].

pH neutralization in the presence of carboxylic acids is potentially caused by the extrusion of a base. NMR spectroscopy of spent medium from *C. albicans* grown on lactate, αkg, and glucose will identify molecules possibly responsible for raising the extracellular pH[18,19].

I have established that eliminating the ability to import carboxylic acids decreases fitness when challenged with macrophages, but the mechanism for pathogenesis remains unknown (Fig. 1B). Carboxylic acid utilization could affect a variety of macrophage processes, including pyroptosis, phagolysosome acidification, phagosome + lysosome fusion, and ROS production[6,7,20,21]. These processes will be individually inhibited pharmacologically to determine which processes rescue survival and killing of the carboxylic acid mutants[6,7,20,21]. Additionally, it is currently unknown if *C. albicans* maintains intracellular pH homeostasis during macrophage attack. To understand this process, the pHluorin constructs expressed in *C. albicans* will track pH of phagocytosed cells compared to cells grown in RPMI medium alone[17].

I will determine the *in vivo* fitness of carboxylic acid mutants using a well-established mouse model for disseminated candidiasis that is often used in our lab[7]. *Jen1*Δ*, jen2*Δ*, jen1*Δ*jen2*Δ*,* complemented strains, and wild type will be inoculated via tail vein injection, and mean time to moribund status (MTMS) and fungal burden of kidneys, spleen, liver and brain will be monitored at fixed time points.

Subaim 1b. Explore indirect mediators of carboxylic acid utilization

A mutant library screen revealed several genes associated with pH and/or growth defects on the dicarboxylic acid αkg[13]. Some of the mutants identified, summarized in Table 1, have clear roles in carboxylic acid metabolism whereas others, such as Pep8 and Ali1, have no obvious role and will be the focus moving forward. Strikingly, every mutant identified is significantly attenuated in the macrophage model.

Table 1. Mutants with pH defects in medium containing αkg

Gene	Glu	αkg	Cell wall	Morphology	Function
ALI1	+	+	+	+	NADH-UbQ Oxidoreductase
COX4	+	+		+	Cytochrome c oxidase
CPH1		+	+	+	Morphology regulator
CWT1			+	+	Cell wall regulator
KIS1		+		+	Snf1 signaling complex
PEP8		+	+	+	Retrograde vesicular transport
SIN3	+	+		+	Transcriptional corepressor

"+" denotes either slow growth on the indicated carbon source or an aberrant phenotype observed in cell wall function or morphology[13,26,43–45]

Pep8 is a putative retrograde transport protein, similar to *S. cerevisiae* Pep8 which is involved in translocating membrane components from the endosome to the trans-Golgi network[22]. Although this protein is not characterized in *C. albicans,* other retrograde transport components have been studied. Liu *et al.* report that mutants defective in retrograde transport are susceptible to cell wall and oxidative stressors and are attenuated in the oral thrush mouse model[23]. Additionally, these mutants display an upregulation in cell wall remodeling enzymes controlled by the Crz1 pathway, likely to compensate for an aberrant cell wall. *pep8Δ* is significantly attenuated when challenged with macrophages and displays growth and pH defects on αkg, succinate, and pyruvate. I hypothesize that *pep8Δ* has a defective retrograde transport system, resulting in a decrease in membrane-localized proteins required for carboxylic acid-driven pH neutralization and cell wall remodeling. First, I will evaluate the retrograde transport system in the *pep8Δ* mutant by monitoring vacuole morphology with a fluorescent stain and assessing transport function by detecting secretion of Kar2 via Western blot, which is only secreted when retrograde transport is defective[23]. Next, I will evaluate the cell wall integrity for oxidative and cell wall stressors, as described in Liu *et al.* The cell wall of *pep8Δ* will be evaluated via electron microscopy (EM) and fluorescent microscopy to quantify cell wall thickness and cell wall components such as β-glucan and chitin[24,25]. Transcript levels of cell wall remodeling enzymes, Crz1, and carboxylic acid import and metabolic enzymes will be measured by qRT-PCR. Cell surface shaving with trypsin digestion coupled with mass spectrometry will quantify surface-exposed proteins in *pep8Δ* and WT[26]. Pep8 may be involved in the transport of TCA/glyoxylate cycle intermediates between organelles. GFP-tagging Pep8 will allow for tracking its localization to multiple organelles, including mitochondria, vacuoles, peroxisomes, and Golgi[23,26–29].

Ali1 is described as a cell membrane-bound putative NADH oxidoreductase reported to be critical for filamentous growth and virulence in both macrophage and mouse models[26]. Similar to retrograde transport mutants, *ali1Δ* is also susceptible to oxidative, osmotic stress, and cell wall stress, suggesting that cell wall integrity is compromised and interrelated with defects in carboxylic acid utilization. I will evaluate the cell wall components in the same manner as described for *pep8Δ*. *ali1Δ* has severe growth defects on all non-fermentable carbon sources tested, suggesting that the TCA cycle may be nonfunctional due to defects in pH homeostasis, an imbalance of NAD^+/NADH, or an ineffective respiratory chain. Although Ali1 has been detected in the cell membrane, it may also localize to mitochondria to contribute to respiration. GFP-tagging Ali1 will allow for tracking its localization to either the mitochondria, cytosol, or cell membrane[26,28]. NAD/NADH imbalances will be measured to detect faults in respiration activity and pHluorin will monitor intracellular pH in the *ali1Δ* mutant[17,30].

Expected Outcomes

Several bases could possibly be extruded from the cell to increase the pH. For Jen2 and dicarboxylic acid utilization, byproducts of catabolism are expected to be identified in the spent medium via NMR. Bicarbonate is a likely molecule, for it is a byproduct of the glyoxylate cycle and is known to raise the pH of blood and urine[31]. The same base may also be responsible for pH neutralization in the presence of monocarboxylic acids, but if this is the case, the intracellular pH of the cytosol and vacuole (measured with pHluorin constructs) are expected to decrease to compensate for the alteration of extracellular pH in the *jen1Δ* mutant.

Since *C. albicans* appears to sense monocarboxylic acids, deletion of Gpr1 is expected to eliminate the pH neutralization in the presence of lactate and pyruvate. It is likely that the associated Gα protein and the downstream transcription factor Crz1 are also required for pH neutralization.

The carboxylic acid import mutants are expected to regain virulence when pyroptosis and phagolysosome acidification are inhibited, which would suggest that carboxylic acid utilization induces pyroptosis and raises

phagolysosome pH to promote pathogenesis. Since the carboxylic acid mutants are defective at surviving within macrophages, I expect to observe similar defects in the mouse model. *Jen1Δ* and *jen2Δ* are expected to display significant defects in MTMS and tissue invasion, especially in tissues such as the liver which have high concentrations of carboxylic acids.

Pep8 and Ali1 are both expected to have aberrant cell walls, displaying decreases in cell wall thickness and increases in the immunostimulatory β-glucan cell wall component. Pep8 is expected to contribute to the transport of necessary membrane components for cell wall remodeling and carboxylic acid utilization, whereas Ali1 is likely critical for functional respiration in mitochondria and maintaining redox balance.

Potential Problems and Alternative Approaches

Although NMR is highly sensitive, identification of the extruded base may prove difficult. It is possible that Jen2, a putative proton symporter, effectively raises the extracellular pH by importing protons. If this is the case, then NMR will reveal no extruded base, and metabolism will not be required to alter the pH. If no bases can be identified by NMR, RNA-sequencing of *C. albicans* grown on αkg, lactate, pyruvate, and glucose will potentially identify transporters responsible for extruding bases like bicarbonate.

It is possible that Gpr1 is not involved in pH neutralization with monocarboxylic acids, or it may only sense lactate (and not other monocarboxylic acids). If this is the case, I will take advantage of mutant libraries to screen for mutants that are defective at neutralizing the pH in the presence of lactate and pyruvate. The RNA-sequencing experiment proposed above may also identify additional monocarboxylic acid sensors.

Aim 2: Determine the physiological adaptations upon exposure to host-relevant carbon sources

Several studies have underscored the importance of alternative carbon sources for *C. albicans* pathogenesis[7,11,13,32,33]. The Brown lab has focused on the impact of the monocarboxylic acid lactate, which is present in the mouth, GI tract, vagina, and potentially macrophages. Lactate induces several advantageous adaptations in *C. albicans.* The cell wall is remodeled to mask β-glucan, resulting in an altered host cytokine response, decreased phagocytosis, and increased resistance to stressors and antifungals.[14,24,32,34] I hypothesize that other host-relevant carbon sources may elicit similar adaptations to promote *C. albicans* survival within the host. This aim will focus on identifying the effects of host-relevant alternative carbon sources most likely to be involved in pathogenesis: αkg, GlcNAc, and amino acids.

Research Design

C. albicans responds to lactate by upregulating cell wall synthesis genes regulated by the Crz1 transcription factor, resulting in a remodeled cell wall and enhanced immune evasion, but it is currently unknown how *C. albicans* responds to other carbon sources[14]. After allowing *C. albicans* to reach mid-log phase when grown in the presence of lactate, αkg, GlcNAc, or amino acids, I will quantify cell wall thickness and cell wall structure with EM[34]. Additionally, fluorescence microscopy and ELISA assays will quantify specific alterations to specific cell wall components, including total β-glucan, exposed β-glucan, total chitin, exposed chitin, and mannan[25]. qRT-PCR of transcripts for important cell wall remodeling enzymes, such as manosyltransferases, glucanases, and chitinases will determine if the cell wall alterations are a result of changes in gene expression[14].

Growth on alternative carbon sources may also increase resistance to a variety of stressors[14]. *C. albicans* pre-grown on alternative carbon will be tested for resistance to host-relevant stressors such as ROS[35], osmotic, cell wall, and antifungals[34]. Previous studies indicate that lactate increases the resistance to stressors and antifungals in a manner independent of the canonical Hog1 stress response pathway[14]. Stress response in *C. albicans* is complex and involves several pathways, so I will monitor the expression of several stress response and cell wall integrity pathways, including the Crz1, Hog1, Rim101, and Mkc1 pathways with Western and Northern blots.

C. albicans adaptations induced by alternative carbon sources may result in improved fitness when challenged with macrophages. As a result of cell wall remodeling, *C. albicans* is phagocytosed less frequently and alters host cytokine production after pre-growth on lactate, but it is unknown how pre-growth with other carbon sources affect macrophage interactions. I will monitor fungal survival[11], macrophage killing[11], phagocytosis rates[32] (live and UV-killed cells), pyroptosis induction[36], phagosomal pH neutralization[7], and macrophage cytokine production (live and UV-killed cells)[32,37] to elucidate the effects of alternative carbon sources on *Candida*-macrophage interactions. A previous report shows that different growth media affects *C. albicans* virulence in the mouse model[38]. I will utilize the disseminated mouse model described in Aim 1 to assess

the *in vivo* fitness of *C. albicans* pre-grown in the different carbon sources. Additionally, cytokine production in mouse plasma will be measured using ELISA assays, as described[39].

Expected Outcomes

Growth on host-relevant carbon sources is anticipated to induce adaptations that promote survival within the host. This survival advantage is likely a result of alterations to the cell wall, which is the main point of contact for host recognition. Masking β-glucan is expected to occur during growth on all the alternative carbon sources, which allows *C. albicans* to evade the host by preventing binding by the host ligand Dectin-1[40]. This masking of β-glucan will alter *C. albicans* interactions with the host, resulting in decreased phagocytosis rates and altered cytokine production in both live and UV-killed cells. Production of pro-inflammatory cytokines induced by β-glucan, such as TNF-α and IL-6, will decrease if β-glucan masking increases as predicted. *C. albicans* pre-grown on alternative carbon sources are expected to display increased fungal burdens and MTMS *in vivo* compared to glucose-grown cells as a result of immune evasion and increased stress resistance.

The cell wall alterations are expected to coincide with increased resistance to a variety of stressors. Lactate-grown cells display improved resistance to a variety of stressors and antifungals, which we expect to be a result of increased cell wall density measured by EM. This resistance is likely to be independent of classical stress response pathways since growth with alternative carbon sources under optimal conditions is not expected to stress the cells. As a result, I expect protein levels of Hog1, Rim101, and Mkc1 to display insignificant changes in response to different carbon sources. Crz1 levels are expected to increase since it controls the expression of several cell wall remodeling enzymes.

Potential Problems and Alternative Approaches

Cells will be collected once the cultures have reached mid-log phase. This means that incubation times will vary between carbon sources due to variation in growth rates. Addition of glycerol or glucose to all media can partially normalize growth rates. Ene *et al*. demonstrated that the addition of glucose to minimal medium with lactate does not significantly affect phenotypes compared to lactate-only medium. A recent study reported that some cell wall alterations are pH-dependent[25]. To ensure that the affects observed are independent of pH, experiments will be performed in media buffered at pH 7, 6, 5, and 4.

GlcNAc is a strong hyphal inducer that results in cell flocculation and clumping in a liquid culture, making it problematic to normalize cell counts for all the assays. Pre-growth incubation will be shortened to collect samples before clumping becomes too severe.

The host environment is dynamic and intricate, presenting pathogens with varying carbon sources and stressors throughout the course of infection. Pre-incubation with different carbon sources identify sources which confer survival and tissue invasion advantages, but in reality, the process is much more complex. For example, lactate, which is available at high concentrations in the GI tract, may be critical for initial stages of infection, while amino acids are only important at later stages. To differentiate between early- and late-stage infections, I will infect mice with survival-standardized inocula as described by Odds *et al*[38].

Aim 3: Elucidate the impact of abolishing alternative carbon metabolism

Work from our lab and others have suggested that *C. albicans* can utilize three independent carbon sources that result in the alteration of extracellular pH (amino acids, GlcNAc, carboxylic acids). All three alternative carbon utilization pathways elicit pH changes using separate and genetically distinct mechanisms. Mutants that affect any one process impairs fitness of *C. albicans* within a macrophage and some display virulence defects in disseminated candidiasis mouse model, but there exists a possibility of redundancy between these pathways. I postulate that elimination of multiple host-relevant alternative carbon pathways will have an additive effect, significantly reducing virulence by removing its ability to effectively adapt to the mammalian host. To test this hypothesis, I will utilize the CRISPR system optimized for *C. albicans* to eliminate all three carbon pathways[15].

Research Design

As previously stated, a variety of alternative carbon pathways are essential for *C. albicans* survival within macrophages and the host, but the impact of eliminating multiple carbon pathways remains unknown. I will take advantage of the newly-optimized CRISPR gene-editing system to rapidly perform homozygous deletions and complementation of several carbon pathways, as displayed in Table 2. *H-d* refers to three genes required for catabolism of GlcNAc: *HXK1, NAG1,* and *DAC1. STP2* encodes for the transcription factor controlling the import

and catabolism of amino acids, and *JEN1* and *JEN2* are required for the import of carboxylic acids. Mutants will be scored for general *in vitro* defects, such as growth, pH, and filamentation defects in hyphal-inducing serum and minimal media containing the alternative carbon sources in question. It is possible that *C. albicans* upregulates alternative carbon pathways to compensate for the deleted pathways. The macrophage model, as mentioned previously, is an established model to identify virulence regulators for disseminated candidiasis. These mutants (Table 2) will be characterized extensively in the macrophage model to determine which pathways display redundant or additive effects. Mutants will be assessed for filamentation[7], pH neutralization[11], pyroptosis induction[6], and survival defects[11]. Lastly, as mentioned in Aims 1 and 2, the disseminated candidiasis mouse model will assess fitness of all mutants generated. qRT-PCR analysis will assess compensatory effects in the absence of the other pathways both *in vitro* and in the macrophage model.

Table 2. Mutants generated to eliminate alternative carbon utilization pathways

Strain generated	Pathways eliminated
h-dΔ jen1Δ	glcNAc + monocarboxylic acids
stp2Δ jen1Δ	Amino acids + monocarboxylic acid
h-dΔ jen2Δ	glcNAc + dicarboxylic acids
stp2Δ jen2Δ	Amino acids + dicarboxylic acids
h-dΔ jen1Δ jen2Δ	glcNAc + carboxylic acids
stp2Δ jen1Δ jen2Δ	Amino acids + carboxylic acids
h-dΔ stp2Δ jen1Δ jen2Δ	glcNAc + amino acids + carboxylic acids

Expected Outcomes

Each carbon source is expected to serve as an independent signal for pathogenesis and adaptation to the host. As a result, I expect the deletion of multiple carbon pathways to display an additive effect, with the potential to completely attenuate virulence in the mutant lacking all three pathways. Amino acid utilization has recently been implicated as essential for the induction of pyroptosis by raising the pH of the phagosome[6]. I expect mutants defective in all three pathways, all of which are responsible for pH neutralization, to be severely defective in pyroptosis induction, filamentation, and pH neutralization. As a result, this mutant will display severe defects in fungal survival and macrophage killing, while mutants lacking one or two pathways will display intermediary phenotypes. Each pathway is regulated by different transcription factors and signals, so increased expression to compensate for the absence of other pathways is not expected.

Potential Problems and Alternative Approaches

All three carbon pathways share the common characteristic of promoting pH neutralization. If additive decreases in virulence and fungal survival are not observed, pH neutralization may be the explanation for redundancy. This pH neutralization process may be binary for macrophage interactions, meaning it is either completely functional to promote pathogenesis, or not effective enough to contribute to macrophage survival. If this is the case, I expect blocking of phagosome acidification with bafilomycin will rescue macrophage survival in every mutant[7].

Mutants generated may display general growth defects. If this is the case, I will generate additional mutants to eliminate alternative carbon utilization without deleting catabolic enzymes. *Ngt1Δ* will replace *h-dΔ* to eliminate import of GlcNAc without deleting catabolic enzymes. Addtionally, *ssy1Δ* (amino acid sensor) will replace *stp2Δ* to eliminate the possibility of pleiotropic effects which are possible when deleting a transcription factor. Generating these additional mutants will also verify results obtained with the initial deletion mutants.

Future Directions

Future endeavors will focus on the global implications of alternative carbon utilization. Although disseminated candidiasis is the most impactful and lethal disease caused by *C. albicans*, vaginal candidiasis, oral thrush, and biofilm formation on medical devices and implants are also critical issues deserving of our attention. Lactate and amino acids are found in a variety of host niches, including the mouth and the urogenital tract, suggesting that these carbon sources likely regulate virulence for multiple *C. albicans* infections[41]. Investigating the importance of alternative carbon in the context of vaginal candidiasis and oral thrush is a future interest.

Several *Candida* pathogens upregulate alternative carbon utilization pathways upon phagocytosis, suggesting that the connection between metabolism and pathogenesis is a conserved pathway. Future studies will focus on the importance of alternative carbon metabolism in related species, especially the multi-drug resistant emerging pathogen *C. auris*[42].

In summary, the results obtained from this proposal will not only yield critical and novel findings in the short term, but it will also lay the foundation for the development of future studies. Alternative carbon utilization is a complex, yet critical component of *c. albicans* infections, and understanding this process and how it contributes to host-fungal interactions will reveal important virulence regulators that may be promising antifungal targets.

References

1. Diekema, D., Arbefeville, S., Boyken, L., Kroeger, J. & Pfaller, M. The changing epidemiology of healthcare-associated candidemia over three decades. *Diagn. Microbiol. Infect. Dis.* **73,** 45–48 (2012).

2. Brown, G. D. *et al.* Hidden Killers: Human Fungal Infections. *Sci. Transl. Med.* **4,** 1–9 (2012).

3. Shapiro, R. S., Robbins, N. & Cowen, L. E. Regulatory circuitry governing fungal development, drug resistance, and disease. *Microbiol. Mol. Biol. Rev.* **75,** 213–267 (2011).

4. Sanglard, D. & Odds, F. C. Resistance of Candida species to antifungal agents: molecular mechanisms and clinical consequences. *Lancet Infect. Dis.* **2,** 73–85 (2002).

5. Pfaller, M. A. & Diekema, D. J. Epidemiology of invasive candidiasis: A persistent public health problem. *Clin. Microbiol. Rev.* **20,** 133–163 (2007).

6. Vylkova, S. & Lorenz, M. C. Phagosomal neutralization by the fungal pathogen Candida albicans induces macrophage pyroptosis. *Infect. Immun.* **85,** 1–14 (2017).

7. Vylkova, S. & Lorenz, M. C. Modulation of Phagosomal pH by Candida albicans Promotes Hyphal Morphogenesis and Requires Stp2p , a Regulator of Amino Acid Transport. **10,** (2014).

8. Lorenz, M. C., Bender, J. A. & Fink, G. R. Transcriptional Response of Candida albicans upon Internalization by Macrophages. **3,** 1076–1087 (2004).

9. Rubin-Bejerano, I., Fraser, I., Grisafi, P. & Fink, G. R. Phagocytosis by neutrophils induces an amino acid deprivation response in Saccharomyces cerevisiae and Candida albicans. *Proc Natl Acad Sci U S A* **100,** 11007–11012 (2003).

10. Fradin, C. *et al.* Granulocytes govern the transcriptional response, morphology and proliferation of Candida albicans in human blood. *Mol. Microbiol.* **56,** 397–415 (2005).

11. Miramón, P. & Lorenz, M. C. The SPS amino acid sensor mediates nutrient acquisition and immune evasion in Candida albicans. *Cell. Microbiol.* **18,** 1611–1624 (2016).

12. Vesely, E. M., Williams, R. B., Konopka, J. B. & Lorenz, M. C. N-acetylglucosamine metabolism promotes survival of Candida albicans in the phagosome. **7422. Accepted.**

13. Danhof, H. A. *et al.* Robust Extracellular pH Modulation by Candida albicans during Growth in Carboxylic Acids. *MBio* **7,** e01646-16 (2016).

14. Ballou, E. R. *et al.* Lactate signalling regulates fungal β-glucan masking and immune evasion. *Nat. Microbiol.* **2,** 16238 (2016).

15. Min, K., Ichikawa, Y., Woolford, C. A. & Mitchell, A. P. Candida albicans Gene Deletion with a Transient CRISPR-Cas9 System. **1,** 1–9 (2015).

16. Lorenz, M. C. & Fink, G. R. The glyoxylate cycle is required for fungal virulence. *Nature* **412,** 83–86 (2001).

17. Tournu, H., Luna-tapia, A., Peters, B. M. & Palmer, G. E. In Vivo Indicators of cytoplasmic, Vacuolar, and Extracellular pH Using pHluorin2 in Candida albicans. *mSphere* **2,** 1–14 (2017).

18. Griffin, J. L. Metabolic profiles to define the genome: can we hear the phenotypes? *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **359,** 857–71 (2004).

19. Emwas, A.-H. M. The Strengths and Weaknesses of NMR Spectroscopy and Mass Spectrometry with Particular Focus on Metabolomics Research. in *Metabonomics: Methods and Protocols* (ed. Bjerrum, J. T.) 161–193 (Springer New York, 2015). doi:10.1007/978-1-4939-2377-9_13

20. Van Der Goes, A. *et al.* Reactive oxygen species are required for the phagocytosis of myelin by macrophages. *J. Neuroimmunol.* **92,** 67–75 (1998).

21. Steinberg, T. H. & Swanson, J. A. Measurement of phagosome-lysosome fusion and phagosomal pH.

Methods Enzymol. **236,** 147–60 (1994).

22. Liu, T.-T. T., Gomez, T. S., Sackey, B. K., Billadeau, D. D. & Burd, C. G. Rab {GTPase} regulation of retromer-mediated cargo export during endosome maturation. *Mol. Biol. Cell* **23,** 2505–2515 (2012).

23. Liu, Y. *et al.* Role of retrograde trafficking in stress response, host cell interactions, and virulence of Candida albicans. *Eukaryot. Cell* **13,** 279–287 (2014).

24. Ene, I. V *et al.* Carbon source-induced reprogramming of the cell wall proteome and secretome modulates the adherence and drug resistance of the fungal pathogen Candida albicans. 3164–3179 (2012). doi:10.1002/pmic.201200228

25. Sherrington, S. L. *et al.* Adaptation of Candida albicans to environmental pH induces cell wall remodelling and enhances innate immune recognition. 1–28 (2017).

26. Gerami-Nejad, M., Berman, J. & Gale, C. A. Cassettes for PCR-mediated construction of green, yellow, and cyan fluorescent protein fusions in Candida albicans. *Yeast* **18,** 859–64 (2001).

27. Hsu, J. W., Chang, L. C., Jang, L. T., Huang, C. F. & Lee, F. J. S. The N-Terminus of Vps74p Is Essential for the Retention of Glycosyltransferases in the Golgi but Not for the Modulation of Apical Polarized Growth in Saccharomyces cerevisiae. *PLoS One* **8,** 1–16 (2013).

28. Bambach, A. *et al.* Goa1p of Candida albicans localizes to the mitochondria during stress and is required for mitochondrial function and virulence. *Eukaryot. Cell* **8,** 1706–1720 (2009).

29. Chen, Y. *et al.* The malfunction of peroxisome has an impact on the oxidative stress sensitivity in Candida albicans. *Fungal Genet. Biol.* **95,** 1–12 (2016).

30. Canelas, A. B., van Gulik, W. M. & Heijnen, J. J. Determination of the cytosolic free NAD/NADH ratio in Saccharomyces cerevisiae under steady-state and highly dynamic conditions. *Biotechnol. Bioeng.* **100,** 734–43 (2008).

31. Krieg, B. J., Taghavi, S. M., Amidon, G. L. & Amidon, G. E. In vivo predictive dissolution: Transport analysis of the CO2, bicarbonate in vivo buffer system. *J. Pharm. Sci.* **103,** 3473–3490 (2014).

32. Ene, I. V, Cheng, S., Netea, M. G. & Brown, A. J. P. Growth of Candida albicans Cells on the Physiologically Relevant Carbon Source Lactate Affects Their Recognition and Phagocytosis by. **81,** 238–248 (2013).

33. Naseem, S., Min, K., Spitzer, D., Gardin, J. & Konopka, J. B. Regulation of hyphal growth and N-acetylglucosamine catabolism by two transcription factors in. 631–632 (2017).

34. Ene, I. V *et al.* Cell Wall Remodeling Enzymes Modulate Fungal Cell Wall Elasticity and Osmotic Stress Resistance. **6,** 1–15 (2015).

35. Mayra Cuéllar-Cruz Omar Arroyo-Helguera, Alejandro De Las Peñas, I. C. <Oxidative stress response to menadione and cumene hydroperoxide in the yeast pathogen Candida glabrata>. *Mem Inst Oswaldo Cruz* **104,** 649–654 (2009).

36. Vylkova, S. & Lorenz, M. C. crossm Phagosomal Neutralization by the Fungal Pathogen Candida albicans. **85,** 1–14 (2017).

37. Duque, G. A. & Descoteaux, A. Macrophage cytokines: Involvement in immunity and infectious diseases. *Front. Immunol.* **5,** 1–12 (2014).

38. Odds, F. C., Nuffel, L. V. & Gow, N. A. R. Survival in experimental Candida albicans infections depends on inoculum growth conditions as well as animal host. *Microbiology* **146,** 1881–1889 (2000).

39. Cannom, R. R. M., French, S. W., Johnston, D., Edwards, J. E. & Filler-, S. G. Candida albicans Stimulates Local Expression of Leukocyte Adhesion Molecules and Cytokines In Vivo Author (s): Rebecca R . M . Cannom , Samuel W . French , Douglas Johnston , John E . Edwards Jr . and Scott G . Filler Published by : Oxford University Pr. **186,** 389–396 (2017).

40. Marakalala, M. J. *et al.* Differential Adaptation of Candida albicans In Vivo Modulates Immune Recognition by Dectin-1. *PLoS Pathog.* **9,** 1–12 (2013).

41. Lorenz, M. C. A feast for Candida : Metabolic plasticity confers an edge for virulence. 1–7 (2017). doi:10.1371/journal.ppat.1006144

42. Perlin, D. S., Rautemaa-Richardson, R. & Alastruey-Izquierdo, A. Series Fungal infections 5 The global problem of antifungal resistance: prevalence, mechanisms, and management. *Lancet Infect. Dis.* **3099,** 1–10 (2017).

43. Noble, S. M., French, S., Kohn, L. A., Chen, V. & Johnson, A. D. Systematic screens of a Candida albicans homozygous deletion library decouple morphogenetic switching and pathogenicity. *Nat. Genet.* **42,** 590–8 (2010).

44. Uhl, M. A. *et al.* Haploinsufficiency-based large-scale forward genetic analysis of filamentous growth in the diploid human fungal pathogen C.albicans. *EMBO J.* **22,** 2668–2678 (2003).

45. Moreno, I. *et al.* Characterization of a Candida albicans gene encoding a putative transcriptional factor required for cell wall integrity. *FEMS Microbiol. Lett.* **226,** 159–167 (2003).