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CHARACTERIZATION OF *ATO* GENES IN ALTERNATIVE CARBON METABOLISM

by

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CHARACTERIZATION OF *ATO* GENES IN ALTERNATIVE CARBON METABOLISM

A

DISSERTATION

Presented to the Faculty of

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In Partial Fulfillment

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For the Degree of

DOCTOR OF PHILOSOPHY

by

Heather Ann Danhof

Houston, Texas

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Characterization of the *ATO* Gene Family in Alternative Carbon Metabolism

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As a commensal colonizer and opportunistic pathogen, *Candida albicans* is the most clinically important human associated fungus. Systemic infection carries an unacceptably high mortality rate of ~40% in the growing population of immunocompromised individuals. Macrophages are important innate immune cells that limit the niches in the human body in which *C. albicans* can persist through phagocytic removal. However, following phagocytosis *C. albicans* readily escapes from the immune cell by differentiating into filamentous hyphae, a process that should be inhibited in the normally acidic phagolysosome. We have shown that *C. albicans* induces germination by neutralizing the phagolysosome. To better understand this process we compared transcript profiles of cells in conditions that promote alkalinization *in vitro* to macrophage phagocytosed cells, which revealing an overlapping set of up-regulated genes, including several members of the poorly understood *ATO* family. This family is greatly expanded in *C. albicans* relative to other fungi and has been implicated in both ammonia release (Ammonia Transport Outward) and acetate metabolism. I hypothesized that the Ato proteins are important effectors of the pH change *in vitro* and in macrophages. Deletion of one of the 10 homologs, *ATO5,* or the over-expression of a dominant negative $ATO1^{G53D}$ allele results in a delay in environmental alkalinization *in vitro,* a defect in hyphal formation. Further, these strains form fewer hyphae after phagocytosis, have a reduced ability to escape macrophages, and reside in more acidic phagolysosomal compartments than wild-type cells. Analysis of an *ato5Δ* $ATO1^{G53D}$

double mutant strain revealed additive *in vitro* defects, similar in magnitude to the *stp2*Δ mutant. Additionally, over-expression of many *ATO* genes in a wild-type background significantly increases alkalinization and ammonia release, strongly suggesting functional overlap between them. In a complementary approach we examined Ato function in *S. cerevisiae* Ato proteins as important to weak acid stress tolerance and cytosolic pH homeostasis; revealing that *ato* mutants are sensitive to weak acid stress and are unable to maintain cytosolic pH homeostasis. This defect was largely dependent upon ScAto1. Taken together, we conclude that Ato proteins are important mediators of the host-pathogen interaction by regulating pH in some host niches.

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Introduction

Candida albicans is part of the human microbiome

Recently large scale genomic studies have greatly expanded our knowledge of human health and disease to be influenced by both the human host and the dynamic microbial community within the host, often called the "human microbiome". The number of microbial cells within an individual is estimated to be up to 10-fold higher than the number of human cells present in the body (1) revealing the importance of understanding the dynamic interactions between microbes and the human host. The microbial communities within an individual are both robust and complex, consisting of bacteria, protozoa, viruses, archaea and fungi which differ in composition depending on the anatomical site in which they reside (2-6). *Candida albicans* is an important human-associated fungus and is often acquired from mother to infant during birth as well as in the hospital setting (7, 8). Following initial colonization *C. albicans* persists within an individual as a benign commensal organism of up to 80% of the healthy population (9-11). This life-long association with the human host necessitates that *C. albicans* must be able to contend with a complex and challenging environment that undergoes continuous changes. Some of the stresses encountered are alterations in the commensal microflora due to exposure to antibiotics, exposure to the host immune system, fluctuations in nutrient type and availability, and a wide range of environmental pH in different anatomical sites. Cellular metabolism drives the adaptation process through the allocation of resources in the form of energy, and the amount of energy available to the cell depends on the type and abundance of nutrients available. Therefore, a competitive advantage is conferred to the organism best equipped to utilize the available resources more efficiently than its competitors and adeptly respond to environmental cues and stresses.

The majority of microbiome studies have been focused on identification of bacterial components, with the ecology and interactions of fungal species only recently beginning to be investigated (12). *C. albicans* can be isolated from a broad range of mucosal surfaces including those of the oral cavity, the gastrointestinal tract, and the genitourinary tract (particularly in females), as well as the skin (6). Due to the impact on human health, the initial focus of studies on the fungal component of the microbiome has been on fungal overgrowth during disruptions of mucosal epithelial integrity, such as inflammatory bowel disease (13), during immunosuppressive conditions or treatments which weaken the epithelium such as chemotherapy (14), or after reduction of bacterial populations by exposure to broad spectrum antibiotics (2). The colonization of anatomically distinct sites in the body and dynamic changes in the bacterial and fungal components of the microbiome predict a broad range of dynamic interactions between microbial species in a variety of microenvironments, and the impacts of these interactions on human and microbial well-being are only beginning to be explored.

In the presence of defined risk factors, *C. albicans* can cause localized mucosal infections such as thrush or vaginitis that are fairly common and easy to treat in healthy individuals. However, disruptions of barrier immunity such as surgery, invasive medical treatments (e. g. indwelling catheters, joint replacement, or heart valve replacement), or burn wounds potentiate more serious systemic bloodstream infections (15). *C. albicans* is the fourth most common cause of hospital acquired blood stream infections (15, 16) and disseminated hematopoietic candidiasis can facilitate the infection of nearly every deep seated solid organ including the liver, kidney, lung, and brain. While nosocomial in nature due to the reduced immune status of the host, these infections are attributed to a shift in the delicate balance between *C. albicans* presence as a benign commensal to an opportunistic pathogen (17).

The innate immune system and recognition of *C. albicans*

Innate immune system function is a key determinant in *C. albicans* disease progression (18). The mucosal barriers, professional phagocytic cells, and the complement system are the principle components of the innate immune system. In general, the innate immune response is mediated by pattern recognition receptors (PRRs) on the surface of immune cells. PRRs in turn recognize specific pathogen-associated molecular patterns (PAMPs), leading to the activation of intracellular signaling pathways in the immune cell. The PRR's and PAMPS that stimulate signaling differ depending on the immune cell type involved.

The epithelium that lines the gastrointestinal tract, in conjunction with the commensal bacterial population, is a critical barrier in preventing fungal dissemination to the bloodstream and other body sites. Epithelial cells contribute directly to immunity by inducing the expression of anti-microbial peptides, such as defensins and histatins, that help control *C. albicans* growth. This barrier immunity is an important defense as the first interaction of *C. albicans* with the host immune system occurs at the epithelial surface (19). Epithelial cells possess the ability to distinguish between commensal yeast that reside on the surface of the mucosa and the more pathogenic hyphal form which poses a threat of cellular damage and tissue invasion. This distinction is critical to the balance of protection against infection and over-stimulation of the immune system (20).

The most studied PRRs are the Toll-like receptors (TLRs), and TLR signaling is important in defending against the major pathogenic fungal species including *C. albicans, Cryptococcus neoformans,* and *Aspergillus fumigatus* (21). In particular, TLR2 and TLR4 along with Dectin-1 and Dectin-2 are critical for the recognition of *A. fumigatus* and *C. albicans* (22).

TLR4 is best known for recognition of bacterial lipopolysaccharide (LPS); however, it has also been identified as important for chemokine signaling in response to *A. fumigatus* infection (23). Importantly, loss of TLR4-mediated signaling increases susceptibility to *C. albicans* infection, reduction in neutrophil recruitment, and decreased chemokine production in mouse models of disseminated candidiasis (24). TLR2 is important for recognition of Gram-positive bacterial peptidoglycan and lipoproteins (25). Additionally, TLR2 recognizes fungal phospholipomannan as well as zymosan inducing macrophage tumor necrosis factor α (TNFα) production (26).

Dectin-1 is a non-classical C-type-lectin receptor recognizing β-1,3-glucans, a principle component of the inner fungal cell wall (21). Interestingly, Dectin-1 recognizes the yeast form of *C. albicans* (and several other fungi (20)) but it does not appear to recognize the hyphal form of *C. albicans,* which has been attributed to less β-1,3-glucan exposure during hyphal growth as compared to budding yeast (27). In contrast, Dectin-2 has been shown to be a low affinity mannose receptor that recognizes *C. albicans* hyphae, although the exact ligand has yet to be elucidated (28). Dectin-2 binding is abrogated in the presence of mannan, though not β-glucan, which suggests the hyphal ligand of Dectin-2 is different from the ligand of Dectin-1 that binds to *C. albicans* yeast form (28).

The phagocytic arm of innate immunity is composed of macrophages and dendritic cells, which are derived from monocytes, and neutrophils; all three of these components contribute to *C. albicans* recognition and protective immunity (18, 21, 29). Dendritic cells are professional antigen presenting cells that provide an important link between innate and adaptive immunity through continual sampling of the environment for foreign cells. In their resting (immature) state, dendritic cells are responsible for uptake, processing, and presentation of cytokine-induced maturation signals. Antigen presentation is a complex process responsible for

instructing the appropriate T cell response to the antigenic signals. This is especially important for responding to pathogenic fungi as dendritic cells distinguish between different fungal morphologies. To accomplish this, dendritic cells express an array of cell surface receptors including TLRs, complement receptors (CRs), Fc Receptors (FcRs), and Scavenger Receptors (SRs), each with unique ligand specificity (30). *Candida* recognition by the C-type lectin receptors MR and DC-SIGN promote fungal phagocytosis followed by migration of the dendritic cell to the lymph node (28).

Neutrophils are an abundant phagocytic cell type that function in concert with macrophages to limit *C. albicans* in the human body. Neutrophils have been demonstrated to be more avidly stimulated by β-1-6-glucan, a more minor component of the fungal cell wall, than β-1-3-glucan (31). The primary opsonin receptors of neutrophils are Fc receptors, which bind to immunoglobulin and β2 integrins, facilitating the binding of these downstream components complement coated particles (32). The phagosome in neutrophils gains antimicrobial properties through fusion with granules and secretory vesicles. These vesicles contain a potent combination of microbicidal peptides and proteolytic enzymes, as well as numerous enzymes that promote pathogen killing. Once active, neutrophils are exceptionally effective at generating reactive nitrogen and oxygen species (ROS). ROS are generated by an NADPH oxidase that transfers an electron from cytosolic NADPH to molecular oxygen. This generates a superoxide anion, which can then be converted to other reactive oxygen metabolites, including hydrogen peroxide and hypochlorous acid. Together these ROS serve as highly effective antimicrobial agents. The clinical importance of neutrophil NADPH oxidase is highlighted by research demonstrating that patients with chronic granulomatous disease, a condition characterized by

defective oxidase function, have increased susceptibility to recurrent fungal and bacterial infections (33).

Additionally, neutrophils can attack *C. albicans* through extracellular secretion mechanisms. In this process, neutrophils extrude chromatin that has been decorated with antimicrobial proteins, known as neutrophil extracellular traps (NETs) (34). These NETs have been hypothesized to be a form of innate response that binds microbes, preventing them from spreading, and functionally increases local concentrations of antimicrobial agents to degrade virulence factors and kill bacteria and fungi (34-36). The primary antifungal component of NETs is calprotectin, a chelator of the divalent cations Mn^{2+} and Zn^{2+}, which sequesters these metals and induces nutrient deprivation in fungi (37).

Macrophages are key components of the innate immune system that are present in virtually all tissues. They differentiate from a common myeloid progenitor cell in the bone marrow that is the precursor of many different cell types, including neutrophils, macrophages, and dendritic cells, among others (38). During development, myeloid progenitor cells undergo a series of maturation steps in the bone marrow that produce monocytes, which are released into the bloodstream. Monocytes leave the circulatory system and infiltrate tissues in response to cytokine signals and then differentiate into functionally distinct (M1/M2) macrophage populations in response to environmental cues and immune signals (38). Once activated macrophages are avid phagocytes that though PRRs signaling coordinate reorganization of the plasma membrane to completely surround the target cell, trapping it in a membrane bound compartment designed to clear it from the body.

After phagocytosis is completed, the initial intracellular vesicle is not antimicrobial in nature. This phagosome undergoes a series of maturation steps through which the cellular machinery necessary for the killing and disposal of internalized microorganisms is acquired. Macrophage phagosomes are trafficked through the endocytic pathway in an organized manner that is directed by a large family of GTPases, the RAB proteins (39). Acquisition of RAB5 on the phagosomal membrane is a widely accepted marker for early stage endosomes (40), RAB5 is then activated by guanine nucleotide-exchange factors (GEFs). Activation of RAB5 then facilitates the recruitment and organization of a series of effector proteins (39). As maturation of the phagosome continues, RAB5 is exchanged for RAB7, an accepted marker of "late endosomes", and through the action of vacuolar sorting protein-39 (VPS39) and RAB7-interacting lysosomal protein (RILP), facilitates fusion with acidic lysosomes (39-41). Following this fusion event lysosome-associated membrane protein-1 and -2 (LAMP-1 and LAMP-2) can be localized to the vesicle surface and this newly formed phagolysosome acquires ATP-hydrolysis-driven proton pumps (V-ATPases) which transport protons across the phagolysosomal membrane resulting in a dramatic decrease in vesicular pH (42, 43). A fully matured phagolysosome is an extremely inhospitable environment designed to kill *C. albicans* through exposure to reactive oxygen and nitrogen species as well as degradation by hydrolytic enzymes including a variety of lipases, proteases, nucleases, and glycosidases that require acidification to function (43, 44). In addition to sequestering the fungal cell through phagocytosis, macrophage activation also stimulates chemokine and cytokine release to recruit additional immune cells to the infection site, providing a bridge to the adaptive immune response (18, 45-48).

When the host is immunocompetent the innate immune system is successful in restricting the sites of *C. albicans* colonization to the mucosal surfaces and maintains the fungal burden at low levels through phagocytic clearance (2). However, impairment of innate immunity allows the fungal cell to proliferate, more easily penetrate the epithelium, and disseminate into the bloodstream resulting in a life threatening systemic infection that carries a mortality rate of ~40% (15, 48) even with administration of anti-fungal drugs. This unacceptably high mortality rate underscores the importance of elucidating *C. albicans* pathogenicity, which is driven by a plethora of virulence factors.

Virulence factors of *C. albicans*

In spite of the myriad of host immune defenses controlling *C. albicans*, it can be isolated from healthy individuals and is the most important human associated fungal pathogen (15). In order to counter these host defenses, *C. albicans* employs a significant repertoire of virulence factors to ensure survival including hyphal morphogenesis, secreted proteases, and adhesins. One defining feature of *C. albicans* is its polymorphism; that is the ability to grow as either as a budding yeast or in various filamentous forms that range from germ tubes to pseudohyphae to true hyphal form (49-51). All of these morphologies are readily observed in host tissues and during interactions with immune cells. Numerous physiological signals trigger hyphal formation *in vitro* including physiological temperature (37°C), increased CO_2 levels, exposure to *N*-acetylglucosamine or serum, and nutrient starvation (35, 52, 53). This diversity among signals suggests that hyphal formation is important in a variety of distinct host niches.

The strict requirement of filamentation for *C. albicans* virulence has been debated (54, 55), and yet the idea that morphogenesis is important for pathogenesis is well established (56,

57). Hyphal morphogenesis facilitates tissue invasion and mediates interactions with epithelial cells as well as macrophages (49, 57, 58). Importantly, morphogenesis has been shown to significantly impact recognition by immune cells and rate of phagocytosis, as hyphal cells are phagocytosed at slower rates by macrophages than yeast cells (59). When cultured with immortalized macrophage-like cell lines (RAW264.7 or J774A.1), phagocytosed *C. albicans* cells form robust hyphal projections which distend the macrophage membrane to the point of rupture (60). In striking contrast, filamentation is strongly repressed following neutrophil phagocytosis (32). While not completely understood, the physiological importance of *C. albicans* hyphal morphogenesis to systemic infection is well documented; with the first observations of hyphal formation inside macrophages dating back nearly 50 years (61).

C. albicans strains that are defective in hyphal morphogenesis have significantly reduced virulence in whole animal models, and are more sensitive to macrophage killing in tissue culture (62-64). Further, strains that are constitutively hyphal and unable to grow as yeast are also avirulent in mice (65), strongly supporting the idea that morphogenetic plasticity is necessary for *C. albicans* virulence. Though widely observed, why *C. albicans* robustly forms filaments inside the macrophage was a complete mystery prior to the last decade. Conditions inside a mature phagolysosome should be strongly inhibitory to germination due to the acidic environment and the assault of degradative enzymes, reactive oxygen, and reactive nitrogen species.

While morphogenic plasticity is central to both commensalism and virulence, *C. albicans* also employs a wide range of proteins that contribute to virulence including adhesins, proteases, and superoxide dismutases. Adhesins function to initiate the colonization of a specific host niche. The eight-member *ALS* gene family, named for similarity to Agglutinin-

Like Sequences, are the most studied of *C. albicans* adhesins (66). These GPI-anchored proteins, along with two additional adhesins, Hwp1 and Eap1, decorate the fungal cell wall and contribute to adhesion to the host mucosa and other surfaces (67-69). As components of the cell wall, these proteins are exposed to the environment and have the ability to modulate interactions with immune cells as well as contribute to biofilm formation. The eight Als proteins are highly homologous and redundancy along with allelic variation has complicated efforts to discover their function (66).

Another family of genes that have been shown to be important in mouse models of infection encodes the superoxide dismutases or *SOD,* genes. This six-member gene family is critically important for *C. albicans* oxidative stress resistance. These enzymes function to detoxify radical oxygen species that are encountered during interaction with phagocytes (70). While similar in overall function, these proteins have been characterized to have differential expression patterns and sub-cellular localization. Sod1-3 are localized intracellularly, while Sod4-Sod6 are localized to the cell wall (32, 71, 72). The Sod4 enzyme is expressed only in the yeast cells while Sod5 is expressed in filamentous cells and is important for systemic infection in a mouse model, along with the cytosolic Sod1 (70, 73). Concurrent inactivation of both Sod4 and Sod5 results in significant increases in clearance by phagocytic cells (32, 71), exemplifying their importance to *C. albicans* survival within the human host.

Hydrolytic enzyme production is another important facet of virulence that is conserved across many kingdoms including pathogenic yeasts (74), protozoa (75), and bacteria (76). *C. albicans* produces three significant classes of hydrolytic enzymes including lipases, phospholipase B enzymes, and secreted aspartyl proteases (Saps) (77). Of these three the most comprehensively characterized are Saps, which are found in many pathogenic *Candida* species

but not in non-pathogenic *S. cerevisiae* (78-80). In *C. albicans*, the 10 Sap enzymes have substantially different expression patterns, preferred cleavage sites, and optimal pH conditions (77). This variation facilitates diverse functionality in distinct physiological conditions (77). Sap1-3 have the highest activity at very low pH and are expressed during phenotypic switching from white to opaque cells (80). Conversely, expression of *SAP4-SAP6* is the highest at neutral pH and co-regulated by the transcription factor *TEC1* with the morphological switch from yeast to hyphal form (81). Sap4-6 have been shown to be important in adhesion, tissue invasion, and immune system evasion *in vivo* (77, 82). Sap9 and Sap10 are predicted to be GPI-anchored constituents of the cell wall and Sap9 also has been shown to stimulate neutrophil chemotaxis (82) although the exact function of these proteins has yet to be elucidated. Taken together, the diversity of the Sap proteins promotes *C. albicans* survival and pathogenicity within the host through nutrient acquisition, host tissue adhesion and invasion, and modulation of interactions with immune cells which is a remarkable range of function for a single highly homologous gene family.

Metabolism flexibility in *C. albicans* - links between survival and virulence

In order to proliferate a cell must be able to utilize nutrients from its environment efficiently. The central metabolic pathways of the model fungi *Saccharomyces cerevisiae* have been well-characterized and some aspects of metabolic functionality and regulation are conserved in *C. albicans*. In *S. cerevisiae,* glucose presence induces fermentation and carbon catabolite repression, a process that inhibits pathways needed to utilize other carbon sources for cellular energy, including other sugars, organic acids, lipids, and ethanol (83, 84). This provides an energetic advantage by restricting production of cellular components and enzymes to only those necessary for utilization of the available nutrient (85). Catabolite repression encompasses

two-fold regulation; repression of transcription of genes needed to utilize non-preferred carbon sources, largely through the transcription factor Mig1 (84), and targeted proteolytic degradation of catalytic enzymes for alternative carbon metabolism. These repression mechanisms are so efficient that when glucose becomes scarce, *S. cerevisiae* must undergo a diauxic shift where growth slows and large-scale genetic and proteomic shifts occur to accommodate the use of the available alternative carbon source (84).

However, *Candida albicans* must contend with continual changes in available nutrients in diverse niches of the host; suggesting that rapid metabolic adaptation is necessary for survival. A primary example of differential metabolic requirements in diverse niches is observed in the transcript profiling of *C. albicans* interactions with immune cells, where the cell senses nutrient deprivation during encounters with the host immune system (86, 87). Upon phagocytosis by macrophages, *C. albicans* undergoes massive metabolic reprogramming, involving differential expression of nearly 600 genes, a large majority of which are involved in alternative carbon metabolism (87). Major processes include repression of glycolysis and protein synthesis pathways and upregulation of a plethora of alternative carbon utilization pathways including β-oxidation of fatty acids, the glyoxylate cycle, and gluconeogenesis, with these changes documented at both transcriptional and proteomic levels (87, 88).

Much work has been done to elucidate the *in vivo* significance of this metabolic reprogramming to *C. albicans* virulence. Strains with mutations in key metabolic enzymes of alternative carbon metabolism have been tested in mouse models of disseminated candidiasis. Isocitrate lyase, encoded by the gene *ICL1*, is crucial for the function of the glyoxylate cycle in *C. albicans.* Mutation of this gene results in significant attenuation of virulence (89, 90). Similarly, loss of key enzymes in the gluconeogenesis pathway, Fbp1 and Pck1, resulted in

decreased virulence (90, 91). These investigations demonstrate that alternative carbon metabolism is critical for the establishment of *C. albicans* infection.

Macrophage phagocytosis induces the *C. albicans* genetic program needed to utilize amino acids as a nutrient, including oligopeptide transporters, amino acid permeases, and degradative enzymes (87). Further, several auxotrophic mutants remain fully virulent in whole animal models, indicating that amino acids (either directly or acquired from the breakdown of peptides and proteins) are sufficiently available within the host (92, 93). Amino acid utilization has been classically studied in terms of nitrogen assimilation rather than as a carbon source, but the transcriptional profile suggests *C. albicans* undergoes carbon limitation (87). Our lab is interested in examining if amino acid utilization also impacted *C. albicans* virulence or fitness within the host as had previously been shown for other alternative carbon utilization pathways.

Amino Acid Assimilation in *C. albicans*

Through *in vitro* studies it has been established that *C. albicans* avidly utilizes amino acids as a sole carbon source, which supports robust growth (94). Amino acid catabolism generates rapid changes in the extracellular pH, with neutralization of medium initially at pH 4 in less than 24 hours, termed “alkalinization”. Additionally, consistent with preference for sugars of *C. albicans*, a rise in pH is not observed in cultures containing significant amounts of glucose (94). However, glucose is in limited supply in the phagosome, indicating that alkalinization may be an important defense mechanism against the acidification of this compartment.

Neutralization of the medium is correlated with the extrusion of ammonia, a strong base, from alkalinizing cells. The ammonia is hypothesized to be derived from the deamination of

amine groups during amino acid catabolism and to be the effector molecule of pH change (94). Neutral pH is a strong signal to induce germination, and our group established *C. albicans* can auto-induce hyphal formation during alkalinization while cells grown in glucose control conditions remain in yeast form (94). Hyphal formation, as discussed above, is important for *C. albicans* virulence, indicating this process may be important in modulating interactions with macrophages.

Genetic screens for mutants defective in alkalinization revealed a strong dependence of alkalinization on amino acid import as mutants in both Stp2, a transcription factor controlling the general amino acid permeases (95), and Csh3, a ER-associated chaperone necessary for amino acid permease folding (96), are completely defective in alkalinization (94). Additionally, mutation of key enzymes of amino acid degradation results in defects in alkalinization, although not as severe as loss of Stp2 or Cph3. Loss of Ach1, an enzyme that converts acetyl-CoA to acetate, results in defects in *in vitro* alkalinization, particularly at early time points (94). Similarly, loss of Dur1,2, a urea amidolyase that generates ammonia and CO_2, delays neutralization the extracellular environment (94). These investigations resulted in a working model of how *C. albicans* achieves environmental alkalinization presented in Figure 1-1 (from (94)) illustrating how *C. albicans* is able to assimilate amino acids into the central metabolic pathways of the cell to generate energy through gluconeogenesis.

Further investigations into the role of Stp2 in alkalinization have strengthened the model presented in Figure 1-1 by revealing that this transcription factor is crucial to *C. albicans* ability to neutralize the macrophage phagolysosome (97). Mutants lacking *STP2* reside in significantly more acidic phagolysosomes than wild-type controls, indicating the observed *in vitro* alkalinization defect had a direct impact on phagosomal pH (97). Further, cells lacking *STP2*

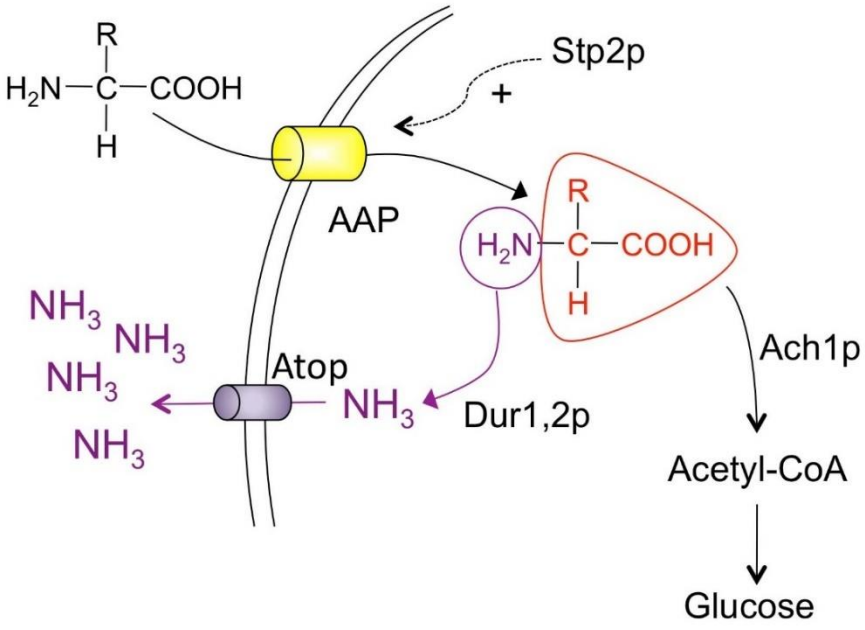

Figure 1-1. Model of environmental alkalinization by *C. albicans*. Under conditions in which amino acids are metabolized as a carbon source, the cell upregulates transmembrane transporters for various amino acids, facilitated by the Stp2 transcription factor. Amino acids are converted into tricarboxylic acid (TCA) cycle intermediates via several routes, many of which require acetyl-CoA production and intracellular transport mediated by acetyl-CoA hydrolase (Ach1p), and all of which remove the amine group(s). In mammals, excess nitrogen is excreted as urea, whereas we propose that in *C. albicans* this is converted into ammonia and CO_2 by urea amidolyase (Dur1,2p) and exported from the cell in a process involving the Ato proteins.

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that were phagocytosed by macrophages showed marked reduction in hyphal formation and ability to escape compared to wild-type (SC5314) cells. This resulted in a higher rate of *C. albicans* killing by RAW264.7 macrophages and a reduction in damage to the macrophage membrane (97). The most conclusive evidence of the importance of *STP2* to *C. albicans* ability to utilize amino acids as a nutrient is shown in the attenuation of virulence in a mouse model of systemic candidiasis (97).

C. albicans sensing of amino acids – the SPS system

Establishment of *STP2* as critical to alkalinization *in vivo* and important for full virulence of *C. albicans* supported our model of alkalinization beyond the *in vitro* phenotypes. However, the source of the amino acid pool utilized in this process *in vivo* remained an open question. Were the amino acids derived from the host, or from intracellular *C. albicans* stores? Our model (Figure 1-1) predicts that the amino acids are scavenged from the host, which is supported by the strong transcriptional induction of secreted aspartyl proteases (*SAP*) and oligopeptide transporter (*OPT*) gene families after macrophage phagocytosis (87). Therefore, the alkalinization defect of *stp2Δ/Δ* cells is most likely a result of reduction in the expression of the necessary amino acid permeases to import amino acids from the extracellular space. However, it is also possible that autophagyand/or vacuolar stores of amino acids could promote *C. albicans* alkalinization. In order to investigate the origin of the amino acids utilized during alkalinization, we examined the role of the extracellular amino acid sensing system. This system is named the SPS system as it is composed of three proteins that form a complex, Ssy1, Ptr3, and Ssy5, that senses extracellular amino acids and activates Stp2 through the proteolytic cleavage of the nuclear exclusion signal (95). As this system functions upstream of Stp2 activation and senses amino acids from the extracellular environment, SPS mutants were ideal

for determining the source of amino acids *in vivo*, as presumably disruptions of the SPS system would not interfere with the utilization of intracellular amino acids

Concurrently with the work presented in this thesis, it has been established that deletion mutants of any of the SPS system components (*SSY1, PTR3* or *SSY5)* result in abrogation of alkalinization and an inability to extrude ammonia, as is seen with the loss of *STP2* (98). Consistent with the hypothesis that host derived external amino acids are driving force of alkalinization, a strain lacking *SSY1*, the external amino acid sensor, phenocopied the *stp2*Δ/Δ mutant defects described above *in vitro* and with reduced fungal survival and increased macrophage cytotoxicity in tissue culture (98). Further, these alkalinization defects were suppressed through the over-expression of a constitutively active *STP2* allele (98), further supporting the role of Stp2 as a master regulator of *C. albicans* amino acid utilization.

Ato proteins as modulators of pH

While much of the model of amino acid utilization (Figure 1-1) has been supported in the above-described work, an important aspect of neutralization remained elusive. How is *C. albicans* excreting ammonia into the extracellular space? Ammonia accumulation in the cytosol of the cell would be toxic (99), suggesting the need for rapidly controlled export from the cell. Mutant analysis of the well characterized ammonium permeases, Mep1 and Mep2, established they were not involved in extracellular alkalinization (94), consistent with previous reports characterizing these proteins as inward ammonium transporters during nitrogen limitation (100). The transcript profiles of alkalinizing cells provided a critical clue as to how *C. albicans* may affect pH change. Several members of a large family of uncharacterized genes predicted to encode transmembrane transporters were up-regulated during alkalinization (Table 1 adapted

from (94)). Homologs of these up-regulated *C. albicans* genes had been identified in the model yeast from which it diverged, *S. cerevisiae,* as involved in the export of ammonia and had hence been named *ATO* genes, for Ammonia Transport Outward (101). *S. cerevisiae* has three *ATO* genes, and expression of all three (*ATO1, ATO2,* and *ATO3)* has been reported during growth on media containing glycerol and amino acids, with expression being correlated to extrusion of ammonia from the cells (102). Deletion of any individual *S. cerevisiae ATO* gene results in an overall reduction of ammonia release and pH change, suggesting that the gene products are not functionally redundant (102).

Table 1. The *C. albicans ATO* gene family			
Gene	ORF[1]	*ach1Δ*[2]	WT + Gluc[3]
ATO1	C3_00920W	79.0	176.9
ATO2	C3_00930W	0.37	0.74
ATO3	C2_06680W	36.6	7.6
ATO4	C6_01430C	16.6	3.6
ATO5	C3_05620W	2.6	6.4
ATO6	C3_05600W	2.1	1.4
ATO7	C2_02470C	0.81	1.6
ATO8	C6_01440C	10.1	9.2
ATO9	CR_00970W	0.39	0.74
ATO10	CR_00930W	0.71	2.1

1. The Orf designations have been updated to the latest nomenclature from the *Candida* genome database from the Orf 19 numbers that were previously published.
2. Fold induction in wild-type cells as compared to the alkalinization impaired *ach1Δ* cells. Numbers in red have >2.5-fold induction.
3. Fold induction in wild-type cells in alkalinizing conditions as compared to wild-type cells in glucose repressed non-alkalinizing conditions. Numbers in red have >2.5-fold induction.

(This table has been adapted with permission from the following article: Slavena Vylkova, Aaron J. Carman, Heather A. Danhof, John R. Collette, Huaijin Zhou, and Michael C. Lorenz. 2011. **The fungal pathogen Candida albicans autoinduces hyphal morphogenesis by raising extracellular pH**. mBio 2(3):e00055-11. doi:10.1128/mBio.00055-11. By American Society for Microbiology policy, (the parent organization for the mBio journal) as an author on this paper, I retain the right to reproduce any and all images published for the purpose of my dissertation.)

Individual functions in ammonia release of *S. cerevisiae* Ato proteins have been investigated using fluorescence microscopy, revealing that all three localize to the plasma membrane, and Ato1 alters its membrane localization pattern depending on pH conditions (103). Intriguingly, Ato1-GFP exhibited patchy membrane localization in medium with pHs above 6, and was diffuse throughout the membrane in medium with pHs below 5, leading to the speculation that not only *ATO1* gene expression but its subcellular protein localization is pH dependent. In contrast, Ato3-GFP membrane localization is insensitive to extracellular pH conditions (103). Further, another *S. cerevisiae* study utilized microscopic fluorescence lifetime imaging (FLIM) and fluorescence resonance energy transfer (FRET) techniques to visualize potential interactions between Ato proteins in the plasma membrane. Oligomers composed of homodimers of each Ato protein, and between Ato1-Ato2 but not Ato1-Ato3 or Ato2-Ato3 were observed (104).

It has been suggested that ammonia release in fungi serves as a quorum sensing-type molecule between yeast colonies, however this idea is controversial and the data has been challenging to reproduce (94, 101, 102, 104, 105). Studies have shown that ammonia release from *S. cerevisiae* colonies is a result of amino acid degradation rather than the presence of exogenous ammonium, as disruption of amino acid permeases (Gap1) but not ammonium permeases (Mep1, Mep2, or Mep3) resulted in loss of ammonia release (106). While the similarities in utilization of amino acids were intriguing, alkalinization of *S. cerevisiae* was rudimentary (on the time scale of weeks) compared to the speed in which *C. albicans* neutralized the pH (hours) (94, 101). One notable difference between these species that might underlie the efficiency of alkalinization is the number of *ATO* homologs in *C. albicans* (10) versus *S. cerevisiae* (3).

Bioinformatic analysis revealed that *ATO* gene families were expanded in pathogenic *Candida* species (*C. tropicalis*, *C. parapsilosis*, and *C. albicans*); and, in fact, the species with expanded *ATO* families (those with four or more) showed significantly enhanced alkalinization relative to the species with fewer *ATO* genes (94). The expansion of this family was intriguing since *Candida* species have been known to expand virulence gene families. This trend is particularly true of cell surface or extracellular proteins, including the *LIP*, *SAP*, *ALS*, and *SOD* gene families that were discussed in the virulence factor sections and are necessary for survival in various host niche (66, 67, 70, 77, 87). Further, transcript profiles of macrophage phagocytosed cells revealed an upregulation of nine of the 10 *ATO* genes, strongly suggesting that they play a role in *C. albicans* interactions with the host (87). Taken together, we hypothesized that Ato proteins would be important effectors of ammonia release and modulate interactions with macrophages. Work to addresses this hypothesis is presented in Chapter 3.

Alkalinization as a defense against weak acid stress

In addition to preventing the activity of hydrolytic enzymes in the macrophage phagolysosome, *C. albicans* may also benefit from phagosomal neutralization by inhibiting the uncontrolled influx of weak organic acids into the cytosol disrupting pH homeostasis. In fact, maintaining cytosolic pH in the presence of organic acids would be critical for *C. albicans* survival given the numerous interactions with various bacteria in the gastrointestinal tract that produce organic acids as a by-product of metabolism. Therefore, there is a potential role for environmental alkalinization outside the confines of the macrophage phagosome. Interestingly, *ATO* homologs in *S. cerevisiae*, *Aspergillus nidulans* (AcpA), *Yarrowia lipolytica* (Gpr1), and *Escherichia coli* (YaaH/SatP) have been associated with acetate transport and tolerance. However, direct biochemical evidence for their molecular function is lacking (107-111). A

dominant mutant of *GPR1* (an *ATO* homolog) confers sensitivity to acetate at low pH; this mutant has a substitution in an N-terminal motif, FGGTLN, that is highly conserved in most Ato proteins (111-113). A second conserved N terminal motif N-P-[AV]-P-[LF]-G-L-x-[GSA]-F is also broadly conserved across many bacterial and fungal species (114).

Trying to reconcile the disparate phenotypes reported for Ato proteins is technically challenging in *C. albicans* due to the more complex genetic manipulation and the potential for redundancy between the Ato proteins. Therefore, we chose to instead characterize a much more manageable family of three Ato homologs in *S. cerevisiae* for utilization of amino acids and acetate toxicity. These studies will be presented in Chapter 6.

Candida albicans possesses multiple mechanisms to raise extracellular pH

The evidence described above demonstrates *STP2* as a critical regulator of environmental alkalinization; *in vitro* phenotypes are strikingly clear that Stp2, through the activation of the SPS amino acid sensing system, controls the utilization of amino acids as a nutrient. However, disruption of Stp2 function resulted in only mild attenuation of virulence in whole animal models (97), and mutants in components of the SPS system are fully virulent (98) despite the apparent reduction of expression in many key components of amino acid utilization. These findings suggested that perhaps other mechanisms of alkalinization and acid tolerance were utilized *in vivo.*

Weak organic acids can be metabolized by *C. albicans* and have been shown to impact cell wall composition and immune cell recognition (115). Taken together these findings led us to the hypothesis that other mechanisms to alter extracellular pH are utilized by *C. albicans.* Indeed, work presented in Chapter 4 demonstrates that in addition to amino acid utilization, *C.*

albicans possesses alternative mechanisms to raise extracellular pH in a manner that does not involve ammonia release and is genetically distinct from amino acid catabolism.

Significance of these studies:

Candida albicans is the most clinically important human associated fungus. This opportunistic pathogen may cause superficial infections as well as fatal systemic infections such as systemic candidiasis (116). Systemic candidiasis is the 4th-most important hospital-acquired bloodstream infection in the United States with a high mortality rate of ~40% despite antifungal treatment (10, 116, 117). Through recent medical advances the proportion of the world population that is living with a compromised immune system has sharply risen, and with it so has the prevalence of serious fungal infections (10, 15). Therefore, it is important to understand interactions between *C. albicans* and the innate immune system, the initial defense that the pathogen encounters in the mammalian host.

Professional phagocytes including dendritic cells, neutrophils, and macrophages efficiently recognize *C. albicans* and mount a robust and multi-faceted immune response to clear the pathogen. In counterpoint, *C. albicans* has evolved sophisticated mechanisms to both avoid detection and phagocytosis and to facilitate escape from the immune cell. To better understand how *C. albicans* is able to mount this counterattack, transcriptional profiling experiments were undertaken to understand the genomic response to encounters with immune cells (31, 86, 87, 118). These studies were analyzed with the rationale that changes in transcript profiles in different host niches would provide insight into the adaptation mechanisms of *C. albicans,* which subsequent studies in large part have supported.

The interaction between *C. albicans* and macrophages is complex and dynamic and is a key determinant of the progression of an infection. *C. albicans* is very adept at responding to environmental cues and employs rapid adaptation mechanisms facilitating both persistence and

pathogenicity. Previous studies have focused upon elucidation of the role of "true" virulence factors in C. *albicans* such as hyphal morphogenesis, adherence mechanisms, and biofilm formation, as well as secreted enzymes for their impact upon disease progression. While these studies were critically important to understanding the ability of *C. albicans* to cause disease, there remains a fundamental gap in knowledge of the critical metabolic adaptations that *C. albicans* employs. Acquisition of nutrients and the generation of cellular energy through metabolic flexibility has been demonstrated as the driving force for both commensalism and pathogenicity; strains lacking that metabolic pathways needed to assimilate amino acids, fatty acids, and other alternative carbon sources have reduced virulence (87, 90, 91, 119-122).

Recent work has demonstrated that hyphal growth of macrophage phagocytosed cells is a result of neutralization of the phagolysosome (94). The effector molecule of this pH change is ammonia which we believe is generated as a byproduct of amino acid catabolism to generate cellular energy through gluconeogenesis. The integration of metabolic intermediates to stress response by *C. albicans* represents a novel mechanism to promote persistence and pathogenicity within the human host. Work presented here identifies for the first time the role of a large family of putative membrane transporters as important to *C. albicans'* ability to modulate environmental pH and tolerate weak acid stresses, both of which represent significant challenges to survival within the human host. These findings represent the most detailed characterization of this family of proteins to date, and provide a platform for future studies to elucidate their biochemical function. This study is of clinical relevance because while broadly conserved in fungi and bacteria, these transmembrane proteins are not present in humans and thereby could represent a new target for needed antifungal drugs to fight disseminated candidiasis.

Chapter Two:

Materials and Methods

NOTE: Portions of this chapter are result of work published in 2015: Heather A. Danhof and Michael C. Lorenz "**The *Candida albicans ATO* gene family promotes neutralization of the macrophage phagolysosome**." *Infect Immun* 83:4416 –4426. doi:10.1128/IAI.00984-15. I am the first author for this publication and was responsible for preparing the original manuscript and conducting the experiments described in this paper. I have permission to reproduce any and all of this manuscript, in print or electronically, for the purpose of my thesis in accordance with the American Society for Microbiology (publisher of *Infection and Immunity*) "Journals Statements of Authors' Rights."
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Materials and Methods

Strains and growth media

C. albicans strains were grown in standard conditions in YPD medium (1% yeast extract, 2% peptone, 2% glucose). For growth on plates, 2% agar was added to the medium. To select for nourseothricin-resistant (Nou^R) transformants, 200 μg/ml of nourseothricin (Werner Bioagents, Jena, Germany) was added to the YPD agar plates (123). Alkalinization experiments were performed in glucose-free minimal yeast nitrogen base (YNB) medium with allantoin as the nitrogen source (0.17% yeast nitrogen base, 0.5% allantoin) supplemented with 2% casamino acids as the sole carbon source (YAC medium). Ammonia release was measured on solid media of the same formulation containing 2% agar.

S. cerevisiae strains were grown in standard YPD medium as described above or defined medium containing yeast nitrogen base (YNB) medium with allantoin as the nitrogen source (0.17% yeast nitrogen base, 0.5% allantoin, 2% gluocse) supplemented with Synthetic Complete supplement mixture lacking uracil (Sunrise Science Products) to facilitate the expression and propagation of uracil based plasmids. Acetate toxicity experiments were performed by inclusion of indicated amounts of acetic acid and adjusting the pH of the medium with sodium hydroxide to 4. For microscopy experiments either SD-ura pH 4 as just described or a formulation where glucose was replaced as the carbon source by amino acids and glycerol were utilized as the control media.

Strains used are listed in Table 1. *C. albicans* strains lacking *ATO5* were generated using the SAT-flipper method as described previously (123). Briefly, 300 bp of homology immediately to the 5' or 3' of the *ATO5* open reading frame were amplified by PCR and cloned between the *Kpn*I/*Xho*I and *Sac*I/*Sac*II sites of pSFS1. The resulting *SAT1*–FLP cassette was

used to transform *C. albicans* SC5314 strain by electroporation with selection on YPD-Nou plates. Cassette integration was confirmed in the selected candidates via PCR. To remove the nourseothricin selection marker, the mutant strain was induced to excise the deletion cassette with 1% BSA in YNB medium for 3 days and the Nou^S colonies were selected. This process was repeated to generate the independently-derived homozygous deletion mutants HDC27 and HDC28 (*ato5*Δ*::FRT/ato5*Δ*::FRT*). Complementation of the mutant strain used plasmid pHD-9, which was generated by cloning the *ATO5* open reading frame with 700 bp of 5' UTR between the *Mlu*I and *Xho*I sites of pAG6, a *SAT1*-marked version of CIp10 (97). This plasmid was linearized with *Stu*I and used to transform SC5314 or *ato5*Δ mutant cells to generate the strains *HDC33* (*ATO5/ATO5 RPS10/rps10:Clp10-SAT1*) and *HDC29* and *HDC30* (*ato5*Δ*::FRT/ato5*Δ*::FRT RPS10/rps10::Clp10-ATO5-SAT1*), respectively.

Constitutive expression of the $ATO1^{G53D}$ allele was achieved as previously described (94). Briefly, ~1,000 bp of the *ACT1* promoter from pAU34 (124) was subcloned between the *Kpn*I and *Xho*I sites in CIp10 to generate pHZ116. Then, the $ATO1^{G53D}$ mutation was generated by site-directed overlap PCR using complementary oligonucleotides with a single mismatch to encode a change of Gly-53 to Asp, analogous to the *Y. lipolytica GPR1-1* mutant originally identified by Barth and colleagues (111), and cloned into pHZ116 to generate pML341. The plasmid was digested with *Stu*I and used to transform CAI4-F2 to uridine prototrophy. Accurate integration at the *RPS10* locus was verified by PCR.

The doxycycline repressible strains were constructed in the THE1 strain background that was generated by Nakayama (125). Briefly, the doxycycline repressible promoter from plasmid p97-CAU1 (125) was amplified and cloned between the *Kpn*I and *Xho*I restriction sites in CIp10 (126) to generate the plasmid pHD72. Subsequently, the *ATO* genes were PCR amplified and

cloned between the *Xho*I and *Mlu*I restriction sites in pHD72. Plasmids were sequence verified and digested with *Stu*I and used to transform THE1 to uridine prototrophy. Accurate integration at the *RPS10* locus was verified by PCR.

Alkalinization and ammonia release assays

Alkalinization experiments were performed as previously described (94, 97), using YAC at pH 4.0, as described above. *C. albicans* cells were grown in YPD medium overnight and diluted to OD_{600} = 0.2 in the alkalinization medium. Cells were incubated at 37^0C with aeration for up to 24 hours. Growth was measured via optical density at 600 nm culture pH was measured using a standard pH electrode and cellular morphology was scored by analyzing photomicrographs of at least 150 cells per condition. Experiments were performed at least in triplicate and the data was analyzed using Prism 5.0 (GraphPad) software.

Ammonia release by *C. albicans* cells during alkalinization was assessed using acid traps as previously described (94). In brief, cells were grown in YPD medium overnight, washed in dH_2O and resuspended at an OD_{600} of 1.0 in dH_2O. Cells were spotted onto solid YAC medium at pH 4.0; reservoirs containing 10% citric acid were affixed to the petri dish lid directly underneath the colonies. Cells were incubated at 37^0C and samples from the acid trap collected at 24, 48 or 72 hours after initiation of the experiment. Ammonia was quantified using Nessler's reagent, as described (94, 127). Experiments were performed in triplicate.

Macrophage cytotoxicity assay

C. albicans toxicity on macrophages was assessed using CytoTox96 Non-Radioactive Cytotoxicity assay (Promega) as previously described (97). Briefly, RAW264.7 macrophages were seeded at $2.5x10^5$ cells per well of a 96 well plate in phenol red-free RPMI and incubated overnight at 37°C, 5% CO_2. *C. albicans* cells were grown to log phase in YPD media, washed in

PBS and co-cultured with macrophages at a 3:1 ratio for five hours. Calculation of lactate dehydrogenase (LDH) release by infected macrophages was then determined according to the manufacturer's protocol relative to maximum LDH release from lysed macrophages and corrected for spontaneous release of LDH by the macrophages or *C. albicans* alone. The experiment was performed in triplicate.

Hyphal formation of phagocytosed *C. albicans*

To assess the interaction of single *C. albicans* cells with macrophages we seeded 2.5 $X10^5$ cells RAW264.7 macrophages to glass coverslips in a 12-well plate and incubated them overnight at 37°C, 5% CO_2.. *C. albicans* cells were grown in YPD medium overnight, diluted 1:100 in fresh medium and grown for 3 hours at 30°C. Cells were then washed in dH_2O and stained with 1 μM 5-Carboxytetramethylrhodamine (Molecular Probes) for 15 minutes, washed 2 times with PBS and resuspended in RPMI medium (Hyclone). 3×10^6 *C. albicans* cells were co-cultured with the macrophages at 37°C for 2 hours. The co-cultures were then washed twice with Phosphate Buffered Saline (PBS) and images of the *Candida*-macrophage interaction were taken using an Olympus IX81 automated inverted microscope. Images from 100 phagocytosed cells per experiment were analyzed using SlideBook 6.0 software. Percent hyphal morphogenesis during phagocytosis was calculated by obtaining percentage of phagocytosed cells using the following formula: (germ tubes + hyphal cells/total amount of cells)×100. Experiments were performed in triplicate.

End-point dilution assay

C. albicans survival during interaction with the RAW264.7 macrophages was assessed as previously described (63, 97). Briefly, macrophages were seeded at $2.5x10^4$ cells/well in 96 well plates and grown overnight at 37°C with 5% CO_2. *C. albicans* cells were grown to log

phase then washed in dH_2O and resuspended in fresh RPMI medium. $1x10^4$ cells/well were added to wells with or without macrophages, followed by six serial 1:5 dilutions. After 48 hours, microcolonies of *C. albicans* in wells in which individual colonies could be distinguished were counted using an inverted microscope. Results were presented as the ratio of (number of colonies in the presence of macrophages/number of colonies without macrophages) x 100. The experiment was performed in triplicate.

Lysotracker Red assay

Assays were performed as previously reported in Vylkova and Lorenz (97). RAW264.7 macrophages were seeded onto glass coverslips in 12-well tissue culture plates at $5x10^5$ cells/ml and allowed to adhere overnight 37°C in 5% CO_2. Next, 1 mM Lysotracker Red DM99 (Molecular Probes) was added to fresh RPMI media and incubated for 2 hours. *C. albicans* cells were grown overnight in YPD, diluted 1:100 in fresh YPD, and grown for three hours at 30°C. Cells were then washed in dH_2O, stained with 1 μM FITC for 15 minutes, and washed in PBS to remove excess dye. Control cells were heat killed by incubation for 60 minutes at 65°C. Cells were diluted to $1x10^6$ cells/ml in phenol red-free RPMI medium and co-cultured with macrophages for 60 minutes. Cultures were stained with Calcofluor white (35 μg/ml for 30 seconds) to label non-phagocytosed cells, and fixed in 2.7% paraformaldehyde. The co-cultures were then imaged at 60X. To estimate the relative phagosomal pH, signal intensities of both FITC and TRITC were plotted along a line drawn transversely across the short axis of the cell for at least 50 cells per condition using Slidebook 6.0. The average Lysotracker Red (LR) signal intensity was calculated for a region of 10 pixels (1 μm) immediately outside the fungal cell, whose boundary was determined by the slope of the FITC signal.

***In vivo* virulence assay**

A murine model of disseminated *C. albicans* infection was performed as described in (90). *C. albicans* cells were grown to mid-log phase in YPD, washed, and resuspended in phosphate buffered saline (PBS) at $5x10^6$ cells/ml. Ten female ICR mice (weighing 21 – 25 grams) per strain were inoculated via tail vein injection with 100 µL PBS containing 10^6 *C. albicans* cells. Mice were monitored at least twice daily and euthanized when moribund. All mouse experiments were performed under protocols approved by the Animal Welfare Committee of the University of Texas Health Science Center at Houston (protocol HSC-AWC-12-099).

Fluorescent Microscopy to monitor cytoplasmic pH of *S. cerevisiae*

This assay was performed utilizing the Cellasic microfluidic system. The Wild-type strain (Σ1278b), *ato* Δ/Δ/Δ (LY342), *ato1Δ* (LY338), *ato2Δ* (LY351), or *ato3Δ* (LY355) strains expressing pHluorin (B728), were grown over night in SD-ura medium, then sub-cultured in SD-Ura medium until mid-log phase and then loaded into a Cellasic microfluidic chamber. SD-ura, pH of 4 (unbuffered) was flowed over the cells continuously located in a microfluidic chamber on the stage of Olympus IX81 microscope at 30°C with ambient oxygen levels. The media was changed to SD-ura+0.25% Acetate, pH 4 and a live action movie was taken of the cells utilizing an Olypus IX81 microscope. At 7 minutes the medium was reverted to the intitial SD-Ura pH 4 without acetate medium. SlideBook 6 software was utilized for image analysis.

Acetate toxicity assays

Wild-type Σ1278b (LY40) and *ato* Δ/Δ/Δ (LY342) were incubated in SD-uracil pH4 with the addition of increasing amounts of acetate for one hour and plated for colony forming units (CFU) on rich media (YPD). Results are reported as means +/- the standard deviation of triplicate experiments. Statistical analysis was performed using Prism GraphPad 6.0 software.

Table 2-1. Strains used in this study			
Strain Name	**Relevant Genotype**	**Complete Genotype**	**Reference**
C. albicans **SC5314**	Wild-type	Prototroph	(128)
SVC17	*stp2Δ*	*stp2Δ::FRT/stp2Δ::FRT*	(97)
HDC27	*ato5Δ*	*ato5Δ::FRT/ ato5Δ::FRT*	(129)
HDC30	*ato5Δ* complement	*ato5Δ::FRT/ ato5Δ::FRT RPS10/rps10::ATO5*	(129)
Can572	*ATO1^{G53D}*	*ura3/ura3 RPS10/rps10::CIp10-ACT1p-ATO1^{G53D}*	(94)
HDC48	Wild-type^{TetR}	*ade2::hisG/ade2::hisG ura3::imm434/ura3::imm434 ENO1/eno1::ENO1-tetR-ScHAP4AD-3xHA-ADE2 RPS10/rps10::CIp10-Tet^{R}*	(129)
HDC44	*ATO1*	*ade2::hisG/ade2::hisG ura3::imm434/ura3::imm434 ENO1/eno1::ENO1-tetR-ScHAP4AD-3xHA-ADE2 RPS10/rps10::CIp10-Tet^{R}- ATO1*	(129)
HDC38	*ATO1^{G53D}*	*ade2::hisG/ade2::hisG ura3::imm434/ura3::imm434 ENO1/eno1::ENO1-tetR-ScHAP4AD-3xHA-ADE2 RPS10/rps10::CIp10-Tet^{R}- ATO1^{G53D}*	(129)
HDC39	*ATO2*	*ade2::hisG/ade2::hisG ura3::imm434/ura3::imm434 ENO1/eno1::ENO1-tetR-ScHAP4AD-3xHA-ADE2 RPS10/rps10::CIp10-Tet^{R}- ATO2*	(129)
HDC40	*ATO3*	*ade2::hisG/ade2::hisG ura3::imm434/ura3::imm434 ENO1/eno1::ENO1-tetR-ScHAP4AD-3xHA-ADE2 RPS10/rps10::CIp10-Tet^{R}- ATO3*	(129)
HDC41	*ATO4*	*ade2::hisG/ade2::hisG ura3::imm434/ura3::imm434 ENO1/eno1::ENO1-tetR-ScHAP4AD-3xHA-ADE2 RPS10/rps10::CIp10-Tet^{R}- ATO4*	(129)
HDC42	*ATO5*	*ade2::hisG/ade2::hisG ura3::imm434/ura3::imm434 ENO1/eno1::ENO1-tetR-ScHAP4AD-3xHA-ADE2 RPS10/rps10::CIp10-Tet^{R}- ATO5*	(129)

HDC43	*ATO10*	*ade2::hisG/ade2::hisG* *ura3::imm434/ura3::imm434* *ENO1/eno1::ENO1-tetR-ScHAP4AD-3xHA-ADE2* *RPS10/rps10::CIp10-Tet^{R}- ATO10*	(129)
HDC49	*ato5Δ* *ATO1^{G53D}*	*ade2::hisG/ade2::hisG* *ura3::imm434/ura3::imm434* *ENO1/eno1::ENO1-tetR-ScHAP4AD-3xHA-ADE2* *ato5Δ::FRT/ ato5Δ::FRT* *RPS10/rps10::CIp10-Tet^{R}- ATO1^{G53D}*	(129)
HDC33	Wild-type-*SAT1*	*RPS10/rps10::CIp10-SAT1*	(129)
HDC31	*ato5Δ-SAT1*	*ato5Δ::FRT/ ato5Δ::FRT* *RPS10/rps10::CIp10-SAT1*	(129)
S. cerevisiae			
LY40	Σ1278b	*ura3-52* MATa	(130)
LY338	*ato1Δ*	Δ*ycr010::G418* ura3-52 MATx	This study
LY342	*atoΔ/Δ/Δ*	*ura3-52* MATa *Δycr010::hygR, Δydr384::hygR* *Δynr002::G418R*	This study
LY351	*ato2Δ*	Δ*ynr002::G418 ura3-52* MATx	This study
LY355	*ato3Δ*	Δ*ydr384::G418 ura3-52* MATx	This study

Table 2-2 Plasmids used in this study		
Plasmid Name	**Description**	**Reference**
pSFS1	SAT1-FLP cassette	(123)
CIp10	Plasmid for integration at RPS10 (URA3 marker)	(131)
pHD59	CIp10 backbone where the *URA3* marker has been replaced with *SAT1*	This study
pHD1	*ATO5* disruption cassette in pSFS1	This study
pHD9	*ATO5* complementation in CIp10-Sat1	This study
p413-SKN	Control plasmid for PCA (*HIS3* marker/Cen)	(132)
p413-SSK	Control plasmid for PCA (*HIS3* marker/Cen)	(132)
P415-YPD	Control plasmid for PCA (*LEU2* marker/Cen)	(132)
p413	Plasmid backbone for PCA assay (*HIS3*/Cen)	This study
p415	Plasmid backbone for PCA assay (*LEU2*/Cen)	This study
pHD31	scATO1 in p413	This study
pHD33	scATO2 in p415	This study
pHD35	scATO3 in p415	This study
pHD38	scGEF1 in p413	This study
pHD39	scJEN1 in p413	This study
pHD40	ScAto1* in p413	This study
pHD42	ScAto1* in 415	This study
pHD44	ScAto2* in 413	This study
pHD46	ScAto2* in 415	This study
pHD48	ScORT in 415	This study
pHD50	ScORT in 413	This study
pHD52	ScTPO3 in 415	This study
pHD53	scATO3 in 413	This study
pHD55	ScRVS in 413	This study
pHD56	ScRVS in 415	This study
pHD61	Ca ACT1p-ATO1 in pHD59	This study
pHD63	Ca ACT1p-ATO2 in pHD59	This study
pHD65	Ca ACT1p-ATO3 in pHD59	This study
pHD66	Ca ACT1p-ATO4 in pHD59	This study
pHD68	Ca ACT1p-ATO10 in pHD59	This study
pHD69	Ca ACT1p-ATO5 in pHD59	This study
pHD72	B526+Tet^R	This study
pHD74	B526-Tet^R-CaATO1	This study
pHD76	B526-Tet^R-CaATO2	This study
pHD78	B526-Tet^R-CaATO3	This study
pHD82	B526-Tet^R-CaATO5	This study
pHD84	B526-Tet^R-CaATO10	This study
pHD94	B526-Tet^R-CaATO1^{G53D}	This study

Chapter three:

Candida albicans ATO genes modulate macrophage interactions by promoting phagosome neutralization

NOTE: Portions of this chapter are result of work published in 2015: Heather A. Danhof and Michael C. Lorenz "**The *Candida albicans ATO* gene family promotes neutralization of the macrophage phagolysosome**." *Infect Immun* 83:4416 –4426. doi:10.1128/IAI.00984-15. I am the first author for this publication and was responsible for preparing the original manuscript and conducting the experiments described in this paper. I have permission to reproduce any and all of this manuscript, in print or electronically, for the purpose of my thesis in accordance with the American Society for Microbiology (publisher of *Infection and Immunity*) "Journals Statements of Authors' Rights."
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Introduction

Candida albicans is an opportunistic pathogen that colonizes the skin, gastrointestinal, and genitourinary tracts of most healthy individuals but also causes a range of diseases from non-lethal mucosal infections such as oral thrush and vaginitis to disseminated hematogenous candidiasis, the latter in immunocompromised individuals (10, 133, 134). As the fourth most prevalent cause of hospital-acquired infection, disseminated candidiasis is very difficult to treat, prolongs hospitalization, and has a mortality rate of ~ 40% (117, 135, 136). The high mortality rates and large health care burden associated with *C. albicans* infection highlight the importance of understanding the physiology, virulence factors, and host-pathogen interactions of *C. albicans*.

The healthy immune system is able to effectively prevent systemic candidiasis, however advances in healthcare have increased the population of individuals surviving despite immune dysfunctions. Conditions that predispose individuals to disseminated candidiasis include hematological malignancies, genetic immune disorders, HIV/AIDS, and iatrogenic interventions, including organ transplantation, chemotherapy, and invasive procedures (134, 137). The interaction between the innate immune system and *C. albicans* is a primary indicator of disease progression, as those with innate immune defects are more susceptible to serious infection(138). Macrophages, along with other professional phagocytes, are key components of the innate immune response to *C. albicans* (138-141). Mice depleted for macrophage function are more susceptible to mucosal and disseminated candidiasis (138, 141). In counterpoint, *C. albicans* has evolved mechanisms to escape phagocytosis and killing by the macrophage, including differentiation into a filamentous hyphal form that facilitates escape and

dissemination. This interaction is highly dynamic and critical for *C. albicans* virulence and therefore remains the subject of intense study (142, 143).

The morphological switch alone does not fully define the response of *C. albicans*, as many studies have revealed large-scale transcriptional reprogramming and proteomic shifts that take place upon phagocytosis including a shift away from glucose metabolism and towards alternative carbon assimilation (31, 32, 87, 88). The importance of metabolic adaptation is underscored by the decreased virulence in animal models of *C. albicans* strains defective the glyoxylate cycle and in β-oxidation (90, 91, 144, 145). Further, carbon source utilization plays a significant role in cell wall composition, stress susceptibility, and phagocyte recognition (146, 147).

Our laboratory has demonstrated that *C. albicans* grown *in vitro* in media that mimics the nutrients predicted to be available after phagocytosis rapidly alkalinizes the extracellular environment (94). This process depends upon utilization of amino acids as a carbon source and leads to the extrusion of ammonia from the cell, which is derived from amino acid catabolism. This secretion of ammonia counteracts the acidification of the phagolysosome, providing a neutral pH signal that induces *C. albicans* to undergo hyphal morphogenesis, thus facilitating escape from the macrophage (97). Further, a mutant lacking the transcription factor Stp2p, which regulates amino acid permeases, fails to alkalinize both *in vitro* and in the phagolysosome and cannot germinate to escape the macrophage unless the phagosome is neutralized by chemical means (94, 97).

The transcript profiles of cells during alkalinization and following phagocytosis share significant similarities, including the induction of multiple genes of the *ATO* family, which at 10 members is greatly expanded in *C. albicans* relative to other fungi. Though named ATO for

Ammonia Transport Outward (and YaaH in bacteria), the molecular function of these plasma membrane proteins is unknown, and there is evidence linking them to transport of acetate in *Saccharomyces cerevisiae*, *Aspergillus nidulans*, and *Yarrowia lipolytica*, as well as release of ammonia in *S. cerevisiae* (94, 101, 102, 107, 112, 114).

We report here that two alkalinization-defective strains, an *ato5*Δ deletion mutant and a strain expressing an $ATO1^{G53D}$ allele originally identified as a dominant negative mutation that confers acetate-sensitivity in *Y. lipolytica* (112), are impaired in several aspects of the macrophage-fungal interaction. These mutations delay alkalinization *in vitro*, leading to a defect in hyphal formation and a reduction in the amount of ammonia released from the cell. Further, both *ato5*Δ and $ATO1^{G53D}$ strains reside in more acidic phagolysosomes than do wild-type cells and as a result form fewer hyphae after phagocytosis and have a reduced ability to escape macrophages. Both *ATO* genes are transcriptionally regulated by Stp2 and a double *ato5*Δ $ATO1^{G53D}$ mutant phenocopies the *stp2*Δ strain. Finally, overexpression of multiple *ATO* genes accelerates alkalinization, indicating that this gene family is an important mediator of the host-*Candida* interaction.

Results

The ability of *C. albicans* to cause invasive infections depends on its dynamic and complex interactions with cells of the innate immune system such as macrophages (21, 142, 148). Strikingly, phagocytosed *C. albicans* cells form hyphae that facilitate their escape, and we have shown that this is induced by a fungal-driven neutralization of the phagolysosome (94, 97). This process is dependent on catabolism of amino acids and is regulated by Stp2p, a transcription factor that activates amino acid permeases (94, 97). We sought to identify whether additional components required for efficient alkalinization also alter interactions with macrophages.

Bioinformatic analysis of *ATO* genes

C. albicans, along with other pathogenic *Candida* species *C. parapsilosis* and *C. tropicalis*, has expanded *ATO* gene families relative to model fungi (94). Previously, a multiple sequence alignment of *C. albicans* Ato proteins was performed and included as part of Dr. Aaron Carman's dissertation (149). However, following that work an additional *ATO* homolog was identified and all of the *ATO* number identifiers were reassigned which complicates referencing this previous work. Consequently, for clarity a new multiple sequence alignment was performed (Figure 3-1) which reflects the current nomenclature from the Candida Genome Database (http://www.candidagenome.org/). Sequence analysis at both the nucleotide and amino acid levels were performed, revealing that on average there is ~50% identity at the nucleotide level across the gene family (Figure 3-2A). The exceptions being *ATO1/ATO2*, *ATO5/ATO6*, and *ATO4/ATO8*, where each pair of genes share ~75% identity (Figure 3-2A). These gene pairs all reside in very close proximity (between 2-5 kB separation) on the same chromosome and therefore are likely duplicated genes. Phylogenetic analysis also supports

ATO1/ATO2, *ATO5/ATO6*, and *ATO4/ATO8* as more related than other members of this gene family as they cluster together on a neighbor joining tree and have the lowest distance values (Figure 3-2C).

At the amino acid level similarity ranges from 30–50% across the gene family with the duplicated pairs having 70-75% identity (Figure 3-2B). Phyre2 analysis of Ato protein sequences (150) predicts both the N- and the C- termini of the proteins are cytosolic, and the protein is dominated by 6 transmembrane spanning helices, denoted with yellow highlighting (Figure 3-1). Protein structure prediction on a more detailed level was not possible due to a lack of validated structures on which to base the prediction; which is consistent with BLAST analysis indicating similarity with only other predicted homologs, none of which have crystal structures.

Amino acid sequences of Ato homologs across clinically relevant *Candida* species were obtained utilizing the *Candida* Genome Database and performing a fungal blast search. These sequences were aligned utilizing ClustalW-Omega multiple sequence alignment and phylogenic tree analysis to assess if there were any striking patterns in the genetic expansions of these protein families. Of note, was how widely dispersed the *C. albicans* homologs are with at least one protein present in most major braches of the cladogram (Figure 3-3). In contrast many of the *C. parapsilosis* homologs cluster closely together.

Figure 3-1. Multiple sequence alignment of *Candida albicans* Ato proteins.

Figure 3-1. Multiple sequence alignment of *Candida albicans* Ato proteins.

Ato protein sequences were obtained from the Candida Genome Database and were aligned utilizing ClustalW-Omega (151). Small and hydrophobic amino acids are displayed in red, acidic amino acids are displayed in blue, basic amino acids are displayed in magenta, glycine and all amino acids containing hydroxyl, sulfhydryl, or amine side chains are displayed in green. The canonical FGGTLN motif of the *ATO*/YaaH gene family is boxed where present. The yellow highlighting at the top depicts predicted transmembrane helices (150).

Ato function is required for efficient environmental alkalinization response in *C. albicans.*

We previously reported that a mutant lacking *ATO5* has a defect in alkalinization in Media 199, a low-glucose tissue culture medium (94). This mutant was generated using the "UAU" method (152) in a strain with several amino acid auxotrophies, which may be problematic given the importance of amino acids in this phenomenon. Therefore, we constructed an *ato5*Δ mutant in the prototrophic SC5314 strain using the SAT-flipper methodology (123). We have subsequently defined a glucose-free minimal media, YNB containing 0.5% allantoin as the nitrogen source and 2% casamino acids as the sole carbon source (YAC), which supports more robust alkalinization (94, 97), and tested both the new *ato5*Δ mutant along with a point mutant in *ATO1* (*ATO1*^{G53D}) which has dominant negative phenotypes in other systems (111). These strains were incubated in YAC starting at pH 4.0, where all strains grew at similar rates (Figure 3-4A). A rapid increase in the culture pH was observed when the wild-type strain (SC5314) was incubated in aerated culture at 37°C (Figure 3-4B), with the pH rising from 4.0 to 6.9 in eight hours, while the *ato5*Δ and *ATO1*^{G53D} mutants were significantly retarded (pH 5.19 and 5.25 respectively) in the same time frame (Figure 3-4B). This lag is overcome by the 24-hour time point, at which all cultures had a pH near neutral. The SC5314-derived strains are more robust than those we used previously, but the magnitude of the *ato5*Δ defect relative to the control strains is similar in both backgrounds (data not shown).

We have attributed this environmental alkalinization to the extrusion of ammonia from the cell (94) and we hypothesized that the *ATO* proteins, which have been proposed to facilitate ammonia export (101, 102), may be important effectors of this release. To

Figure 3-2. Bioinformatic analysis of similarity between *C. albicans ATO* genes.

A.

	Chx 3 *ATO1*	Chx 3 *ATO2*	Chx 2 *ATO3*	Chx 6 *ATO4*	Chx 3 *ATO5*	Chx 3 *ATO6*	Chx 2 *ATO7*	Chx 6 *ATO8*	Chx R *ATO9*	Chx R *ATO10*
ATO1	100	74.69	54.23	55.84	57.76	55.01	57.49	53.18	52	52.91
ATO2	74.69	100	56.28	55.44	56.74	54.75	56.72	52.92	53.89	51.94
ATO3	54.23	56.28	100	57.83	55.61	52.95	53.39	57.33	52.99	57.87
ATO4	55.84	55.44	57.83	100	55.31	54.03	52.16	73.34	48.6	56.34
ATO5	57.76	56.74	55.61	55.31	100	76.87	51.08	55.2	52.21	56.42
ATO6	55.01	54.75	52.95	54.03	76.87	100	50	53.86	50.74	54.67
ATO7	57.49	56.72	53.39	52.16	51.08	50	100	51.86	49.13	56.07
ATO8	53.18	52.92	57.33	73.74	55.2	53.36	51.86	100	49.25	55.63
ATO9	52	53.89	52.99	48.6	55.21	50.74	49.13	49.25	100	41.07
ATO10	52.91	51.94	57.87	56.34	56.42	54.67	56.07	55.63	41.07	100

100 80 55 50 40

Percent Identity

B.

	Ato1	Ato2	Ato3	Ato4	Ato5	Ato6	Ato7	Ato8	Ato9	Ato10
Ato1	100	76.98	51.16	48.81	49.81	47.33	51.36	43.3	35.5	42.96
Ato2	76.98	100	53.88	48.41	48.28	46.18	47.47	44.06	37.87	42.25
Ato3	51.16	53.88	100	41.6	39.92	39.16	40.23	37.79	33.73	37.14
Ato4	48.81	48.41	41.6	100	43.19	42.64	41.77	69.96	30.3	43.66
Ato5	49.81	48.28	39.92	43.19	100	77.86	37.84	40.96	35.54	44.22
Ato6	47.33	46.18	39.16	42.64	76.87	100	35.38	41.54	34.94	40.54
Ato7	51.36	47.47	40.23	41.77	37.84	35.38	100	39	32.53	41.73
Ato8	43.3	44.06	37.79	69.96	40.96	41.54	39	100	29.09	38
Ato9	35.5	37.87	33.73	30.3	35.54	34.94	32.53	29.09	100	8.33
Ato10	42.96	42.25	37.14	43.66	44.22	40.54	41.73	38	8.33	100

100 70 50 30 10

Percent Identity

C.

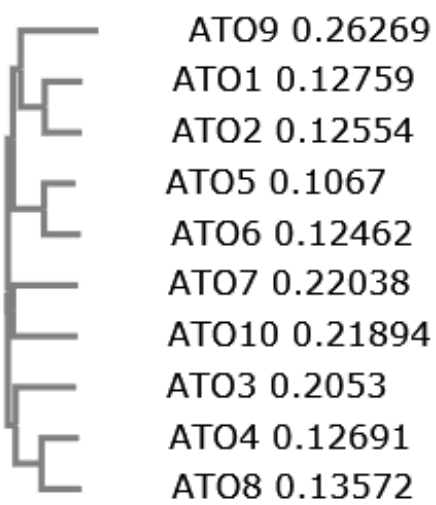

Figure 3-2. Bioinformatic analysis of similarity between *C. albicans ATO* genes.

A) Sequence similarity in CLUSTAL-Omega pairwise comparison of *ATO* genomic sequence and amino acid sequences (B) that were obtained from the Candida Genome Database (151). C) Neighbor-joining phylogenetic tree without distance corrections generated from CLUSTAL-Omega nucleotide alignment (153). Numbers to the right of the gene name indicate the amount of base substitution compared to the nearest neighbors over the length of the gene with lower values indicating high sequence conservation.

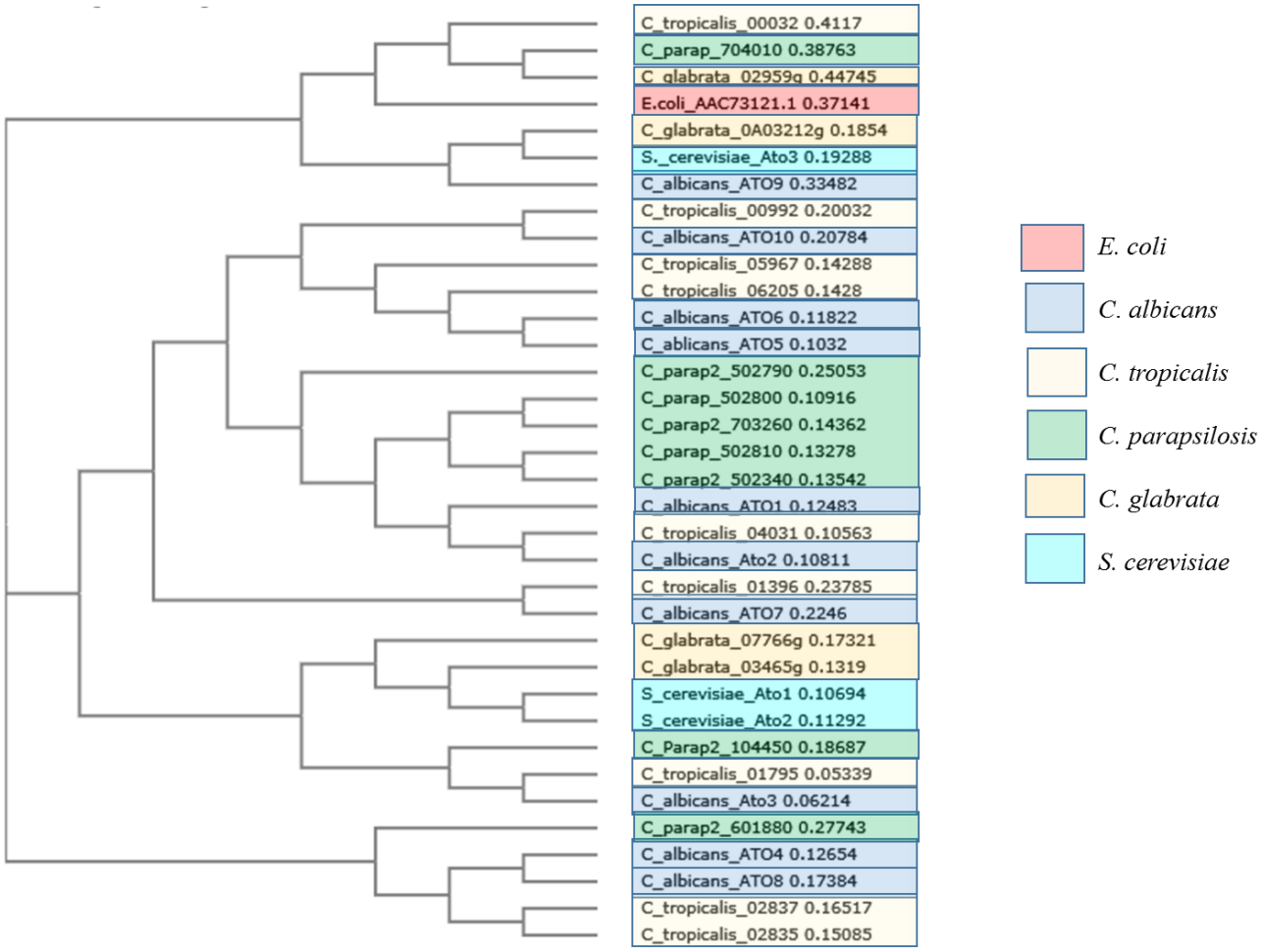

Figure 3-3 Phylogenetic relationships between Ato homologs.

Neighbor-joining phylogenetic tree without distance corrections generated from CLUSTAL-Omega amino acid alignment of Ato homologs in 5 fungal species and *E. coli* (153). Numbers to the right of the protein name indicate the amount of base substitution compared to the nearest neighbors over the length of the gene with lower values indicating high sequence fidelity.

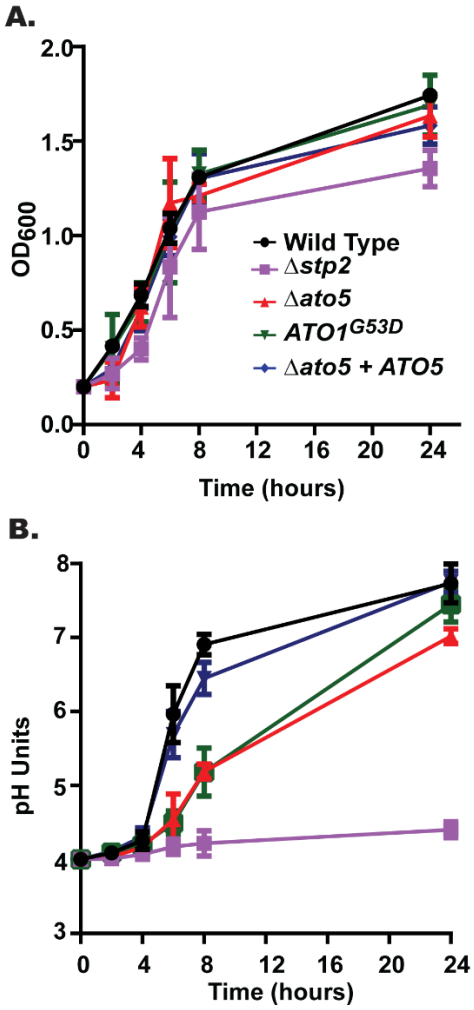

Figure 3-4. Ato proteins promote environmental alkalinization. The wild-type (SC5314), *stp2Δ* (SVC17), *ato5Δ* (HDC17), *ATO1*^{G53D} (Can572), and *ato5+ATO5* (HDC30) strains were incubated in YAC medium initially at pH 4.0 under aerated conditions at 37°C. (A) Growth of the cells was measured by OD600 readings at the indicated time points. (B) pH of the cultures from the same experiments whose results are shown in panel A. Results are reported as mean values +/- SD of triplicate assays.

NOTE: Reproduced from work published in 2015: Heather A. Danhof and Michael C. Lorenz "**The *Candida albicans ATO* gene family promotes neutralization of the macrophage phagolysosome**." *Infect Immun* 83:4416 –4426. doi:10.1128/IAI.00984-15.

test this hypothesis, we performed an ammonia release assay in which colonies were allowed to develop on solid defined alkalinization media (YAC, pH 4) directly apposed across an air interface from an "acid trap" containing 10% citric acid. Ammonia excreted from the colony is converted to ammonium in the acid trap, which can be quantified using Nessler's reagent (94, 154). Detectable ammonia from wild-type (SC5314) and reconstituted *ato5*Δ+*ATO5* cells increased significantly over the 72-hour period (Figure 3-5). In contrast, ammonia release from both the *ato5*Δ and $ATO1^{G53D}$ cells was significantly reduced (Figure 3-4). Ammonia excretion correlated with the degree of alkalinization, with the *stp2*Δ mutant completely deficient, the *ato* mutants intermediate, and the wild-type and complemented strains releasing abundant ammonia.

Our initial formulation of the minimal casamino acid media included ammonium sulfate as the nitrogen source, as is typical in defined yeast media, which we realized may affect our ammonia release results. Indeed, we found that at neutral pH the presence of ammonium sulfate significantly increased the amount of ammonia present in the trap even in the absence of cells, while no ammonia was released from acidic media (Figure 3-5). This may lead to a feedback loop in which ammonia generated by cellular metabolism raises the pH, which in turn liberates ammonia from the media, thus overstating the contribution of the cells. To avoid this, we tested additional nitrogen sources and found that no ammonia was released from cell-free media containing allantoin, urea or amino acids as the nitrogen source, regardless of pH (Fig. 3-6 and data not shown). Allantoin supported optimal growth and all the assays reported here use allantoin as the nitrogen source. The amount of ammonia released is slightly lower on

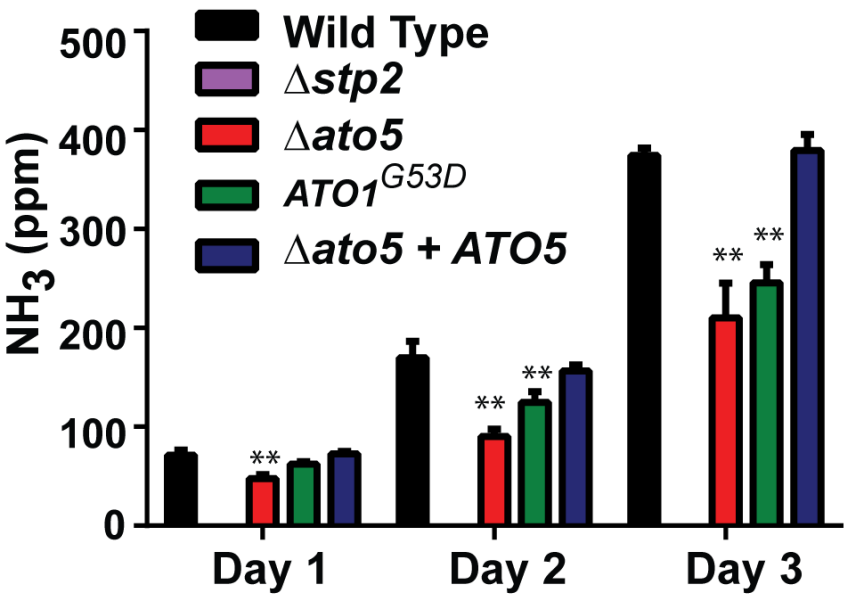

Figure 3-5. Ato proteins promote ammonia release. The wild-type (SC5314), *stp2*Δ (SVC17), *ato5*Δ (HDC17), *ATO1*^{G53D} (Can572), and *ato5*Δ + *ATO5* (HDC30) strains were incubated in YAC medium initially at pH 4.0 in aerated conditions at 37°C. Ammonia released by *C. albicans* cells was collected in a citric acid trap and quantified using Nessler's reagent as described in the Materials and Methods. (** p-value <0.001). Results are reported as mean values +/- SD of triplicate assays.

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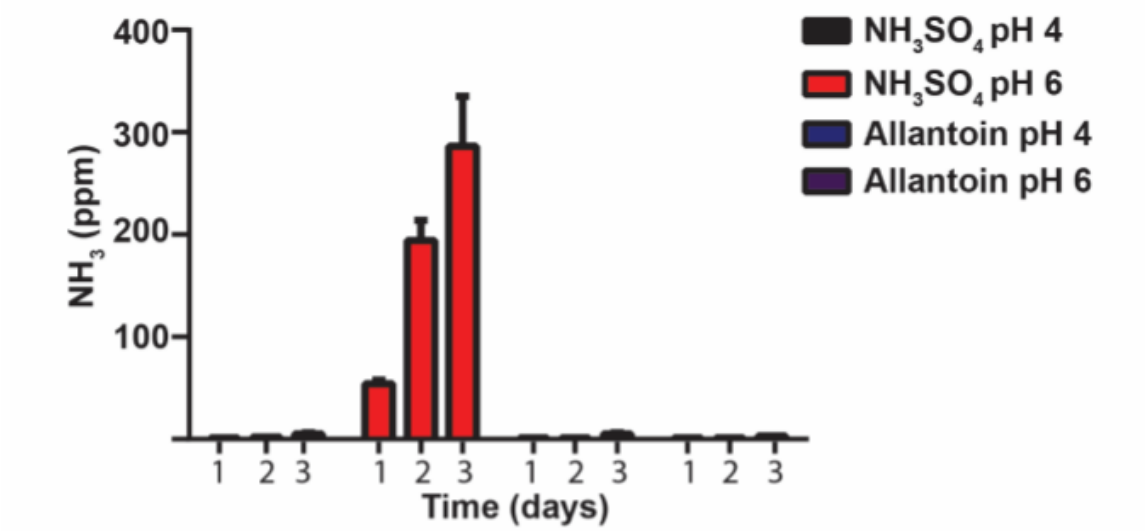

Figure 3-6. Cell free plates containing ammonium release ammonia at neutral pH. Solid agar plates containing yeast nitrogen base, and 0.5% ammonium sulfate or allantoin at pH 4 or 6 were incubated at 37°C for three days. Ammonia release was collected in a citric acid trap and quantified using Nessler's reagent as described in Materials and Methods.

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allantoin than on ammonium sulfate, but the temporal pattern and genetic phenotypes are similar to those we have previously reported and no differences in the rate of pH changes were observed (Fig. 3-4 and data not shown).

Mutation of Ato proteins affects auto-induction of hyphal formation.

The ability of *C. albicans* to undergo a reversible morphological switch from yeast to hyphal form has been shown to be critical to virulence of the organism (62, 155). Neutral pH is a key factor that induces this switch and we have shown that efficient environmental alkalinization is sufficient to promote this morphologic switch (94). Therefore, we hypothesized the *ATO* mutant strains would have an impaired ability to form hyphae due to the defect in alkalinization. The *ato5*Δ and $ATO1^{G53D}$ strains were grown in unbuffered alkalinization media (YAC, pH 4.0) over a period of six hours and were significantly impaired in hyphal formation compared to the wild-type (SC5314) or complemented strains (Figure 3-7A and data not shown). However, when these strains were cultured in the same medium adjusted to pH 7 for two hours no defects in hyphal formation were detected (Figure 3-8 and data not shown), indicating that the mutants were able to respond to neutral pH cues, but could not autoinduce hyphal growth by changing the pH.

We have shown that *C. albicans* blocks the normal acidification of the phagolysosome and that, like *in vitro*, the resulting neutral pH induces hyphal growth; in contrast, an *stp2*Δ mutant does not neutralize this compartment and as a result does not germinate post-phagocytosis (97). We hypothesized that the *ato* mutants would also have a defect in the auto-induction of hyphal formation inside the macrophage. In order to test this, strains were co-cultured with RAW264.7 macrophages for two hours, fixed, and

assessed for hyphal formation microscopically (Figure 3-7B). As expected, the *ATO* mutant strains show a significant reduction in hyphal formation (~50%) while the wild-type (SC5314) and reconstituted strain are nearly all hyphal (94% and 87% respectively; Figure 3-8C). Taken together these results confirm that the ability to efficiently alkalinize the phagolysosome is an important signal for hyphal formation and that the Ato proteins are important effectors of this signaling.

Ato proteins are required for efficient macrophage lysis and escape.

We have shown that the ability to alkalinize the phagosome significantly contributes to the ability of *C. albicans* to escape the macrophage (97). As a result of the alkalinization and hyphal formation defects we predicted that the *ATO* mutant strains would be impaired in the ability to pierce the macrophage and escape. In order to assess this we utilized a lactate dehydrogenase (LDH) release assay as a proxy for macrophage membrane damage after five hours of co-culture of *C. albicans* with RAW267.4 macrophages (97). Co-culture of macrophages with wild-type (SC5314) and *ATO5*-complemented strains resulted in ~75% maximal LDH release (relative to chemically lysed macrophages). In contrast, the *ato5Δ* and $ATO1^{G53D}$ mutants were less able to damage macrophages, with only 50% and 52% of the maximal LDH release while the *stp2Δ* strain released only 42% of the maximum (Figure 3-9A). These data suggest that functional Ato proteins are necessary for efficient escape from the phagosome.

A reduced ability to escape the macrophage might predict that the *ATO* mutant strains are more susceptible to macrophage killing. To address this, we utilized an established end-point dilution assay to assess *C. albicans* survival after phagocytosis (63). In good agreement with the LDH release assay, significantly reduced survival was

seen in the *ato5Δ* (52%) and *ATO1*^{G53D} (62%) strains as well as the *stp2Δ* control strain (45%); in contrast, more than 80% of the wild-type cells survived this interaction (Figure 3-9B). Thus, we conclude that a defect in macrophage escape also leads to an increased ability of the macrophage to clear the pathogen.

Ato5p is necessary for efficient alkalinization of the phagosome.

Taken together, the phenotypes of the *ato* mutant strains strongly suggest that they may have a defect in neutralization of the phagolysosome. To test our hypothesis, we pre-loaded RAW267.4 macrophages with the acidophilic dye Lysotracker Red (LR), which accumulates and fluoresces in acidic organelles, and then co-cultured these cells with FITC-labeled *C. albicans* cells. As expected, heat-killed cells strongly colocalized with LR after 60 minutes of co-culture (Figure 3-10). Conversely, wild-type (SC5314) cells were surrounded by a low-level diffuse LR signal suggesting a more neutral pH (Figure 3-10). *stp2*Δ and *ato5*Δ cells both were frequently contained in acidic compartments (Figure 3-10). To estimate the difference in the pH of phagosomes containing wild-type versus *ato5*Δ or *stp2*Δ cells we utilized the Slidebook 6 image software to quantitate the signal intensity in the FITC and LR channels along a line drawn through the middle of the *C. albicans* cell on the short axis (Figure 3-10B). Plotting the FITC fluorescence intensity clearly delineates the fungal cell (green, Fig. 3-10B-C). The phagolysosomal membrane is tightly apposed to the fungal cell wall, so LR is confined to the lumen in a narrow band immediately outside the cell (notably, in the heat-killed controls, LR accumulates both in the phagosome lumen and in the permeabilized fungal cell), as seen in the sharp rise in LR intensity as the FITC signal is decreasing (Fig. 3-10B-C). This lumenal fluorescence is absent from wild-type and

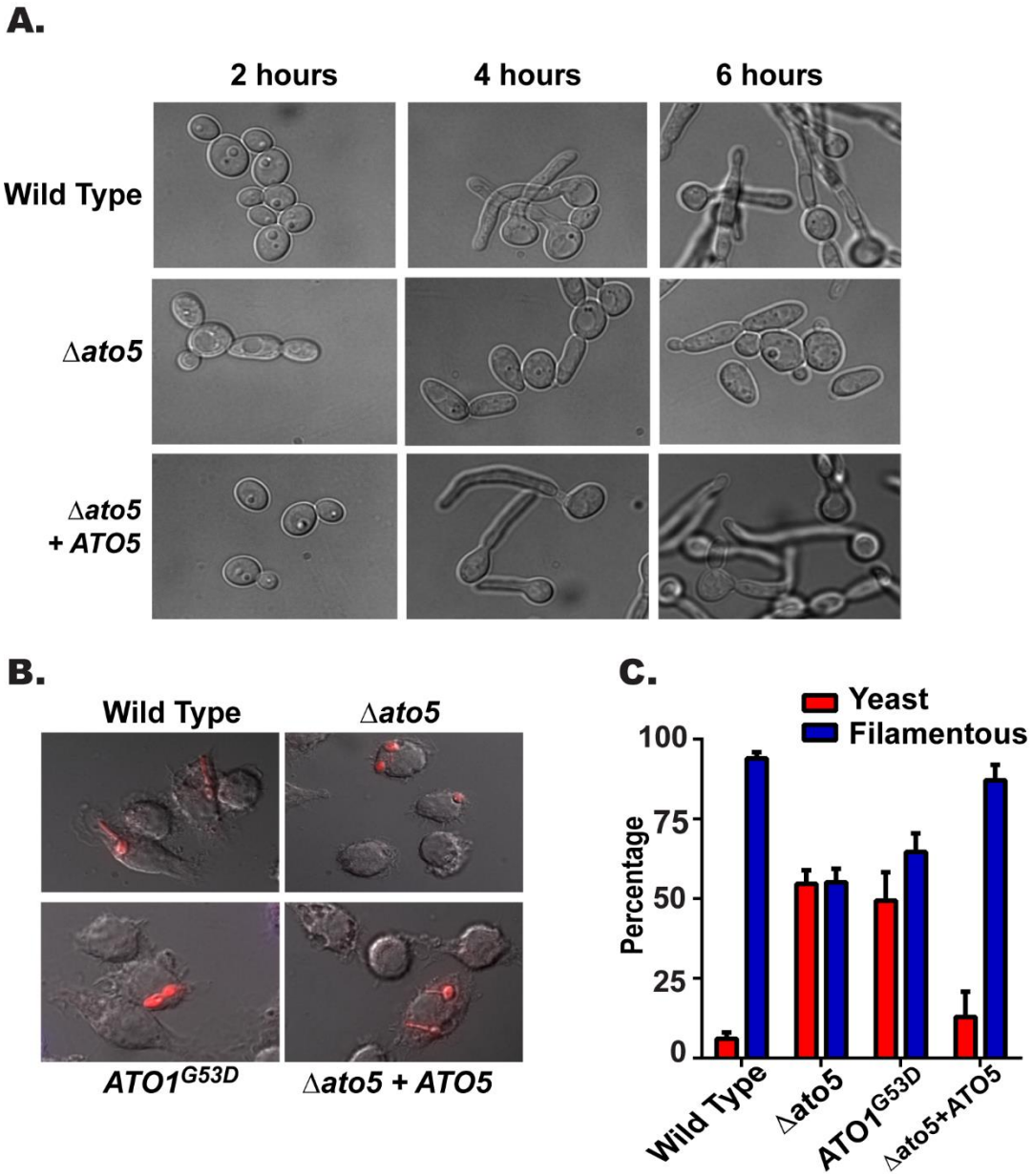

Figure 3-7. Mutation of Ato proteins reduces auto-induction of hyphal formation.

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Figure 3-7. Mutation of Ato proteins reduces auto-induction of hyphal formation. The wild-type (SC5314), *stp2Δ* (SVC17), *ato5Δ* (HDC17), $ATO1^{G53D}$ (Can572), and *ato5Δ* + *ATO5* (*ATO5* complement) strains were assayed for hyphal formation. A) Strains were grown in YAC media for six hours and photographed at 100X. B) Cells were labeled with 5-Carboxytetramethylrhodamine and co-cultured for two hours with RAW267.4 macrophages, fixed, and photographed at 60X. C) Filamentous cells were quantitated in the captured images by counting at least 150 cells per condition. Values are reported as means +/- SD of triplicate experiments.

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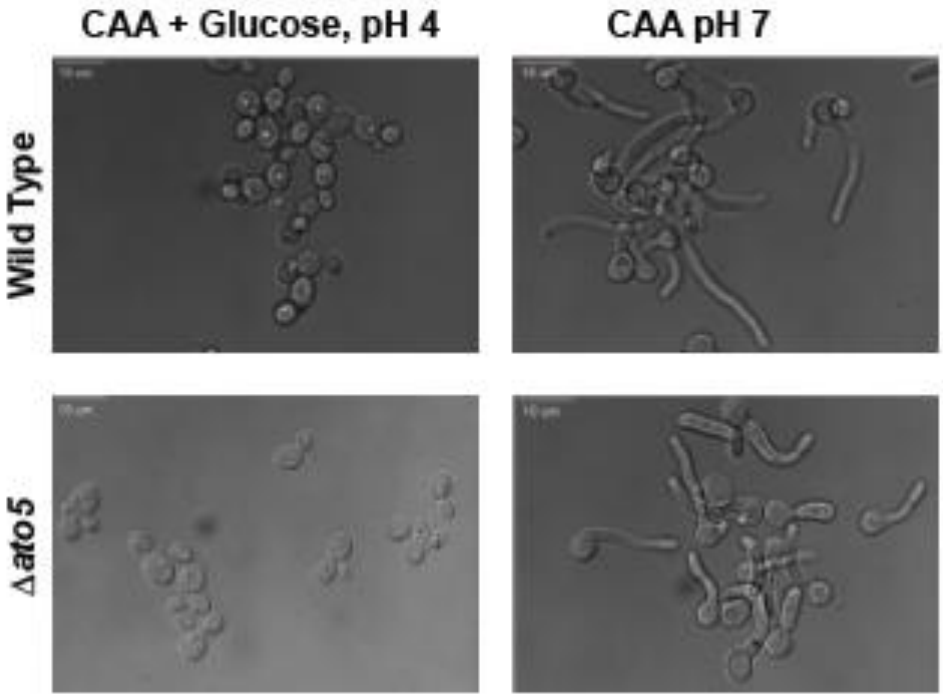

Figure 3-8. *ato5Δ* cells form hyphae at neutral pH. Wild-type (SC5314) and *ato5Δ* strains were grown in CAA pH 4 (with glucose to prevent pH changes), or CAA pH 7, for 4 hours and DIC images were taken. Hyphal formation was quantified.

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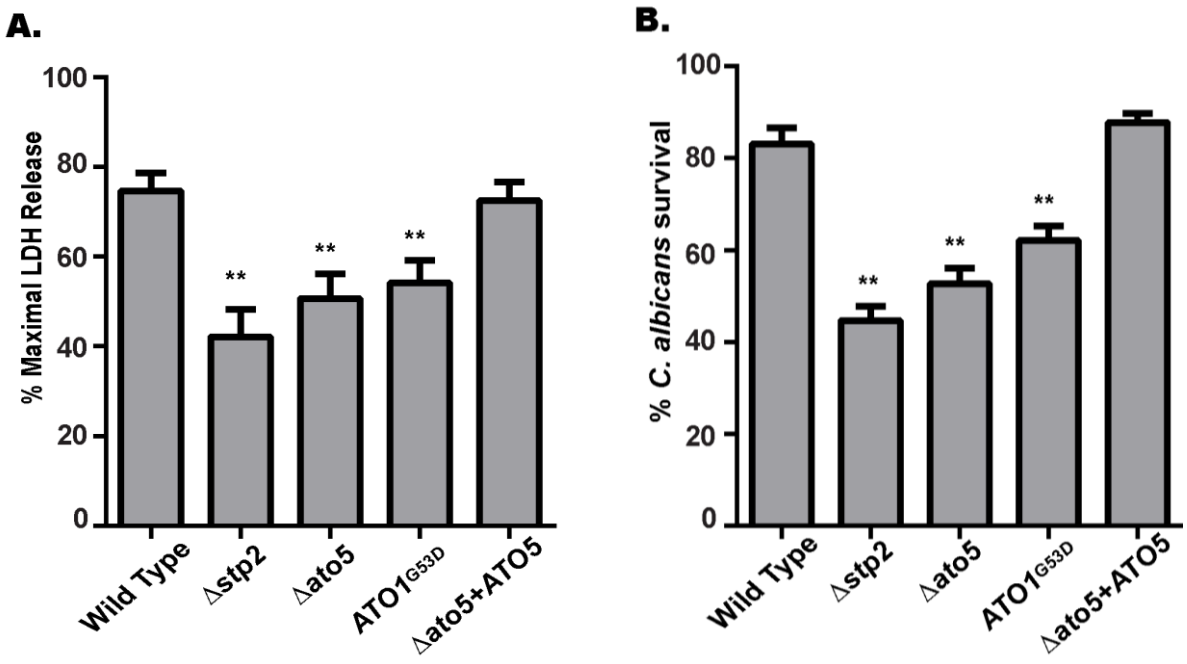

Figure 3-9. Ato proteins are required for efficient lysis of macrophages and proliferation after phagocytosis. A) The wild-type (SC5314), *stp2Δ* (SVC17), *ato5Δ* (HDC17), *ATO1*^{G53D} (Can572), and *ato5Δ* + *ATO5* (*ATO5* complement) strains were co-cultured with RAW264.7 macrophages. Macrophage death was assessed based upon lactate dehydrogenase (LDH) release. B) Strains were co-cultured in the presence and absence of RAW264.7 macrophages for 24 hours. Fungal survival was calculated as the ratio of microcolonies in the presence vs. absence of macrophages (** p-value < 0.001). Values are reported as means +/- SD of triplicate experiments.

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complemented cells, but is readily apparent in *stp2*Δ and *ato5*Δ mutants (Fig. 3-10C). We quantitated the average LR intensity over 10 pixels (1 μm) on each side of the fungal cell (indicated by the dashed regions in Fig. 3-10B). Both the *stp2*Δ and the *ato5*Δ mutant resided in more acidic compartments as indicated by the higher Lysotracker Red signal as compared to the wild-type and complemented strains (Figure 3-10D). From these results we conclude that *ato* mutants occupy an acidic phagolysosome, indicating that the *in vitro* alkalinization defect we have described extends to the phagocyte as well.

Loss of only *ATO5* does not compromise virulence

The macrophage co-culture experiments indicate that *ATO* mutants are less able to tolerate phagocytosis, and suggest this may be an important determinant for virulence of *C. albicans*. We had previously shown that the deletion of *STP2* resulted in a modest but significant attenuation of virulence in this model (97), so we asked if this was true of an *ATO5* deletion strain using the standard mouse tail-vein model of disseminated hematogenous candidiasis (Figure 3-11) and found no statistically significant attenuation in virulence. This result may be explained by the modest alkalinization phenotypes and the potential for functional redundancy with other Ato proteins whose expression may compensate for the loss of *ATO5 in vivo*.

Expression of *ATO* genes is dependent on the transcription factor *STP2*.

Stp2p is a transcription factor that regulates amino acid permeases (95, 97). Given the similar phenotypes of the *stp2*Δ and *ato5*Δ mutants, we asked whether Stp2p had any role in the regulation of the *ATO* genes by using quantitative real time PCR to assess the transcript abundance of *ATO1* and *ATO5* in wild-type and *stp2*Δ strains in alkalinizing conditions. *ATO1*, which is highly induced following phagocytosis and in

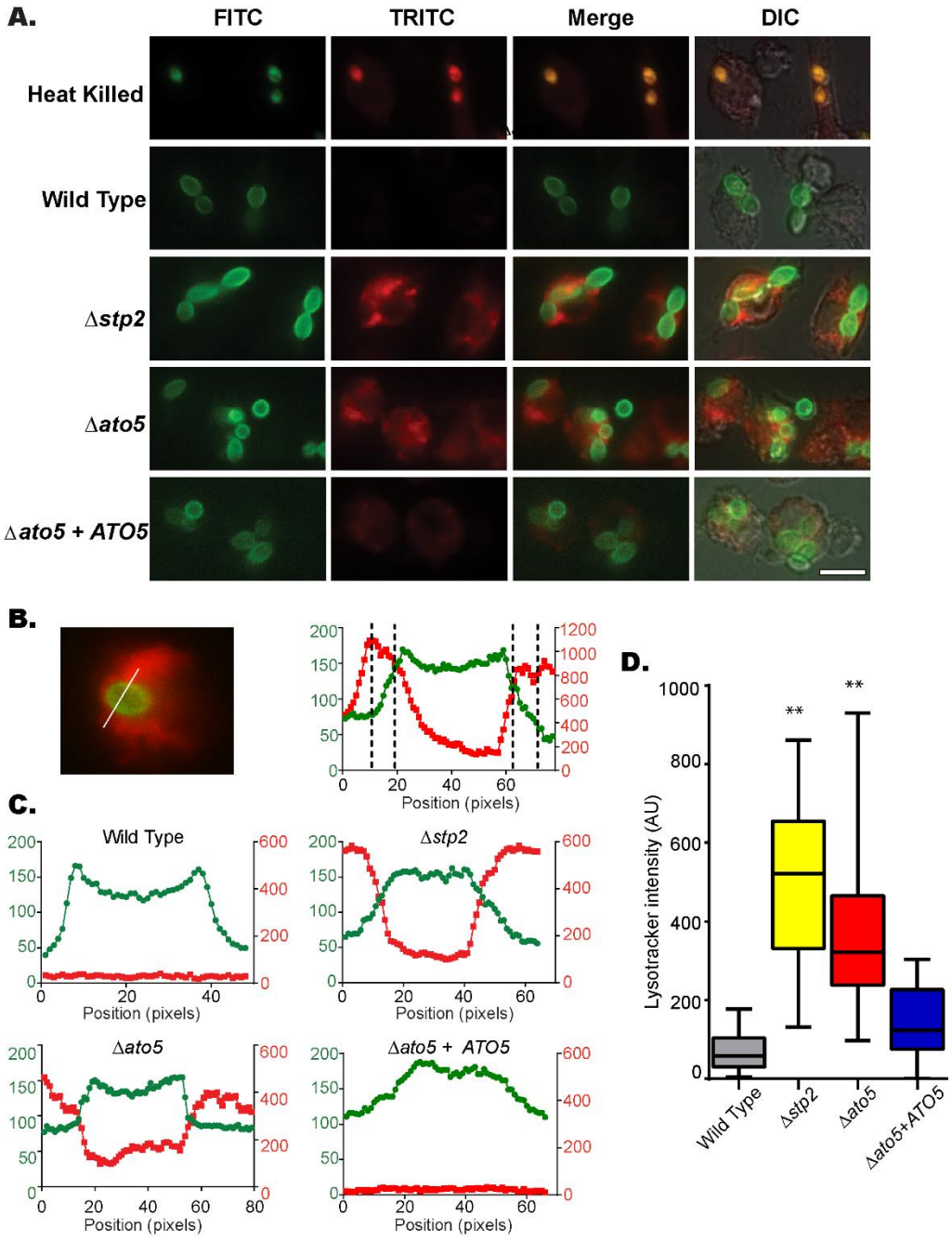

Figure 3-10. Ato5p is necessary for neutralization of the macrophage phagosome.

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Figure 3-10. Ato5p is necessary for neutralization of the macrophage phagosome. A) FITC-stained *C. albicans* cells were co-cultured with RAW264.7 macrophages preloaded with Lysotracker Red for one hour. The co-cultures were then fixed, and imaged at 60X. B**)** Image analysis of the co-cultures described in (A) was performed using Slidebook 6.0 software. Fluorescence intensity for both the FITC and LR channels was plotted along a line drawn through the middle of the *C. albicans* cell on the short axis (10 pixels = 1 μm). Dashed lines indicate the regions adjacent to the fungal cell used to quantitate LR signal. C) Representative plots from each strain. The FITC signal is plotted on the left axis in green and the TRITC signal is plotted on the right axis in red numbers. D) Box (25% - 75%) and Whiskers (minimum to maximum) plot of the average TRITC intensity (** p-value < 0.001). At least 50 cells were counted per strain. All assays were performed in triplicate.

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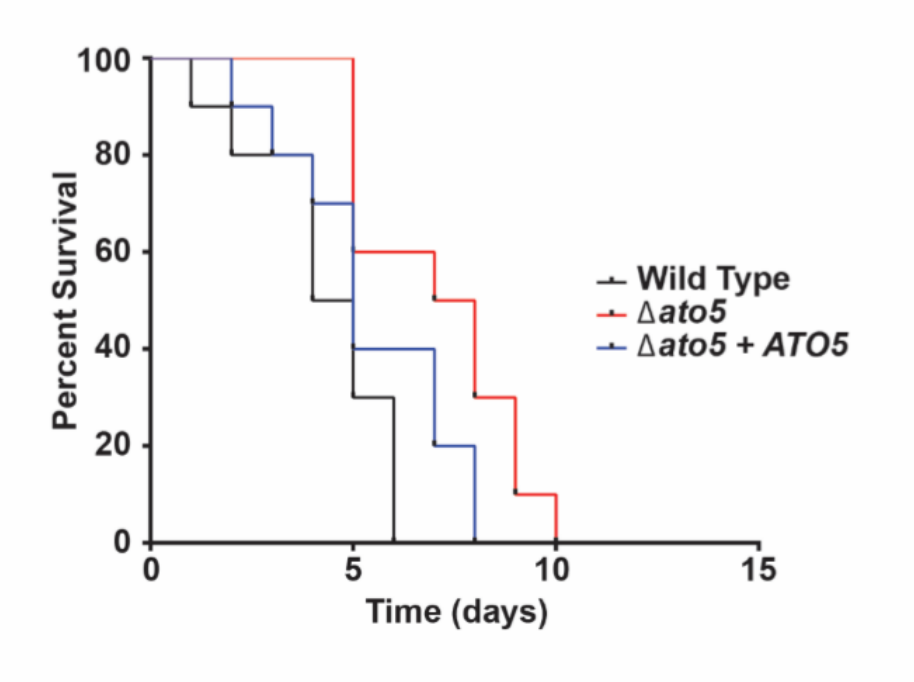

Figure 3-11. Deletion of only *ATO5* does not attenuate virulence. Outbred ICR mice were inoculated via tail vein with $5x10^5$ cells of the wild-type (SC5314), *ato5Δ* (HDC31), or *ato5Δ +ATO5* (HDC30) resuspended in phosphate buffered saline. Ten animals per strain were monitored for signs of infection as defined in the materials and methods.

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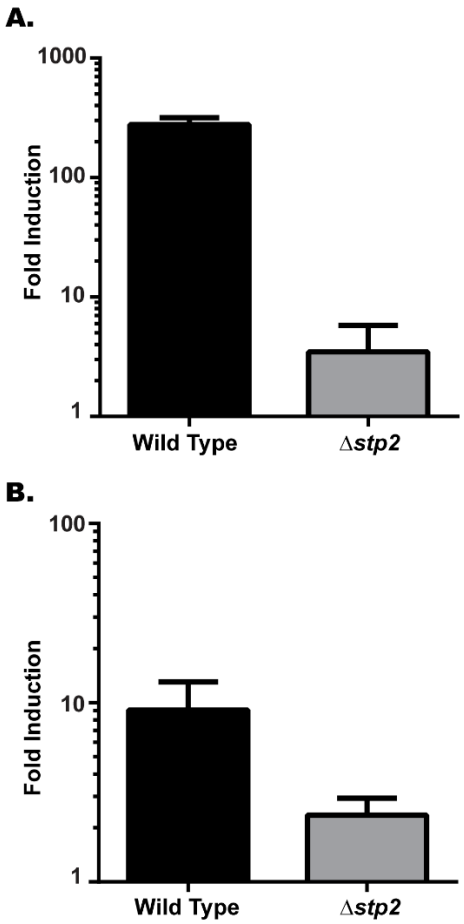

Figure 3-12. *ATO* gene expression is dependent upon *STP2.* Quantitative real-time PCR analysis of cells grown in alkalinization medium. Cells of both the wild-type (SC5314) and *stp2*Δ (SVC17) strains were grown in YAC (alkalinizing conditions) or the same media with glucose added (non-alkalinizing conditions) and the transcript levels of A) *ATO1* and B) *ATO5* expression were determined. Transcript abundance was normalized to *ACT1* and the expression in glucose was set to 1. Data are expressed as means +/- SD of triplicate experiments.

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YNB-CAA (87, 94), was up-regulated 279-fold in wild-type cells as compared to non-alkalinizing conditions. This induction was almost completely abolished in cells lacking *STP2* (Figure 3-12A). Similarly, *ATO5*, was up-regulated 7.5-fold in alkalinizing wild-type cells (Figure 3-12B), but only 2-fold in the *stp2*Δ mutant. Taken together, these results indicate that the *ATO* genes are regulated by *STP2*.

Many *ATO* genes affect *C. albicans* alkalinization

The Stp2-dependence of *ATO* gene expression raised the question of whether heterologous *ATO* expression might suppress *stp2*Δ mutant phenotypes. To test this, we generated *ATO* alleles under the control of the constitutive *ACT1* promoter. Surprisingly, *stp2*Δ strains expressing these alleles failed to grow in YAC media, although growth was unaffected in media containing glucose (Figure 3-13). This suggests that the dysregulation conferred by deletion of *STP2* cannot be suppressed (and might be exacerbated) by overexpression of target genes, perhaps indicative of a careful stoichiometry between Atos and other cellular proteins. To address this, we constructed strains with *ATO* genes under the control of a doxycycline (Dox)-repressible promoter. Overexpression of many *ATO* genes accelerated environment alkalinization compared to the wild-type control (Figure 3-14A) while these strains also released more ammonia (Figure 3-14B-C). Conversely, overexpression of *ATO3* or the dominant-negative $ATO1^{G53D}$ inhibited alkalinization and ammonia release. These data indicate that the *ATO* gene family is broadly involved in the ability of *C. albicans* to alkalinize the extracellular space but that there might be specific interactions between Ato and other proteins that regulate this phenomenon.

Double *ATO* mutations results in additive alkalinization defects

Mutations in *ATO5 (ato5Δ)* and *ATO1* (*ATO1*^{G53D}*)* result in strikingly similar phenotypes and we sought to determine if a double mutant strain would confer a synthetic phenotype. Indeed, *in vitro* alkalinization of the double mutant is drastically inhibited when compared to either single mutant (Figure 3-15), despite near-normal growth in these conditions (data not shown). This phenotype is very similar to an *stp2*Δ strain (Figure 3-15); consistent with this, the double mutant also fails to release ammonia (data not shown). These results provide strong evidence that multiple Ato proteins can facilitate ammonia release, although further studies will be necessary to determine the molecular mechanism through which this process occurs.

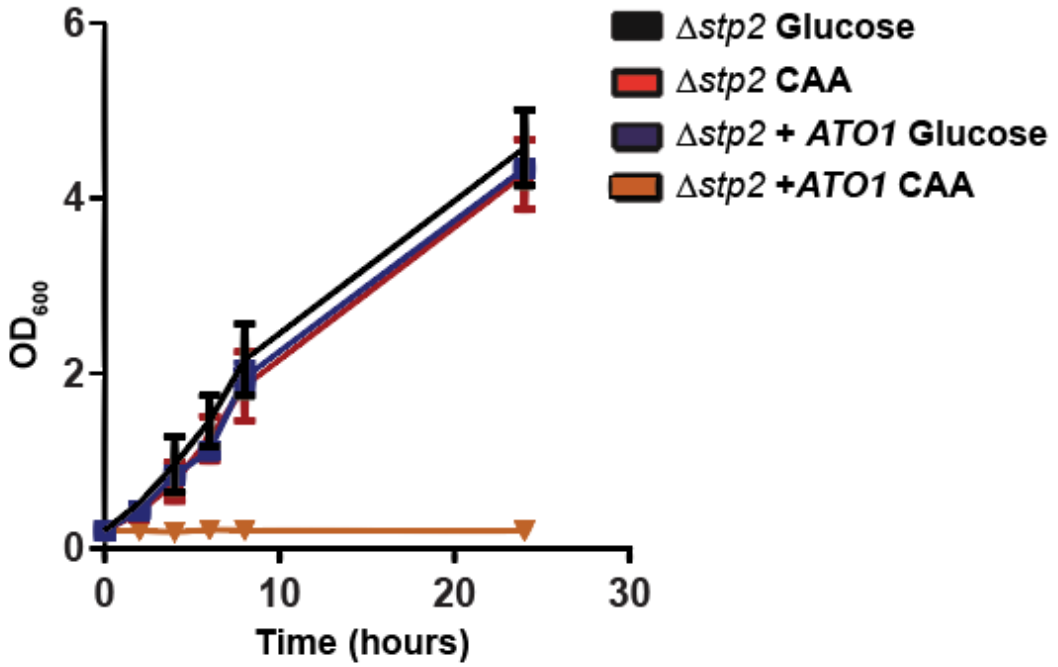

Figure 3-13. *stp2*Δ strains constitutively expressing *ATO* genes fail to grow in casamino acids. The *stp2*Δ (SVC17) strain with an empty vector or with *ATO1* under the control of the *ACT1* promoter were incubated in YAC with or without 2% glucose, starting at pH 4.0 in aerated cultures at 37°C. Growth was measured by optical density at 600nm. A similar YAC growth defect was conferred by overexpressing any of the ATO genes.

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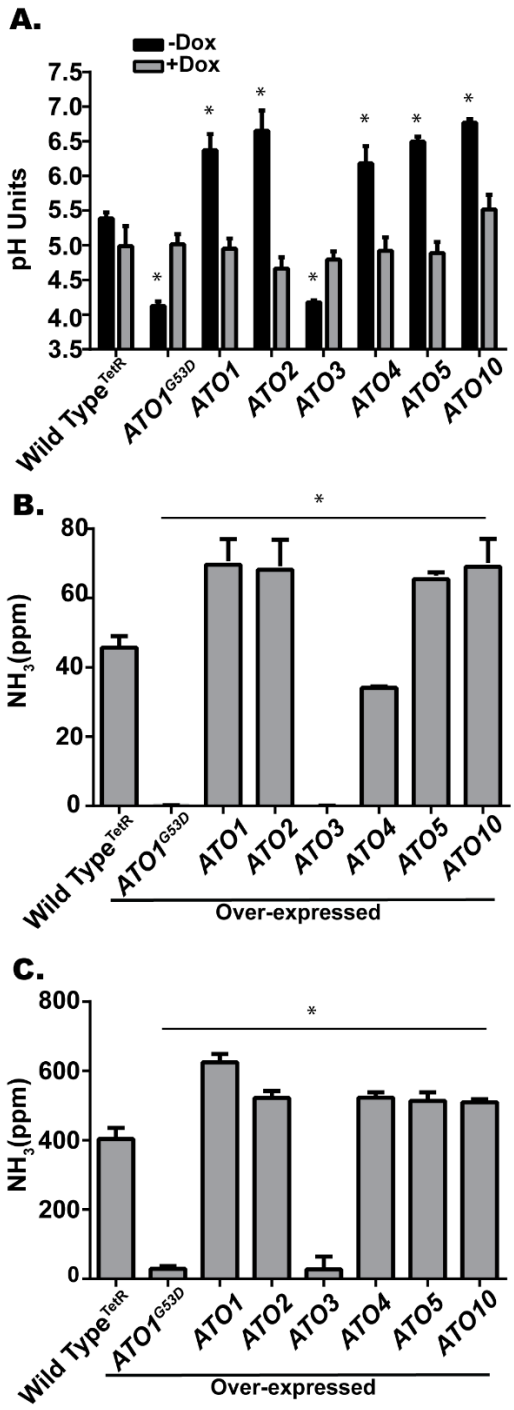

Figure 3-14. *ATO* overexpression alters alkalinization.

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Figure 3-14. *ATO* overexpression alters alkalinization. A) Wild-type (THE1) and strains with *ATO* genes under the control of a doxycycline (Dox)-repressible promoter were incubated in YAC initially at pH 4.0 in aerated conditions at 37°C with and without Dox. A) The pH of the cultures after 8 hours is shown. All overexpression strains (-Dox) are statistically different from the wild-type control (p-value < 0.001). B-C) Ammonia released by *C. albicans* cells during alkalinization on solid media in the absence of Dox after B) 24 or C) 72 hours. Data are expressed as means +/- SD of triplicate experiments.

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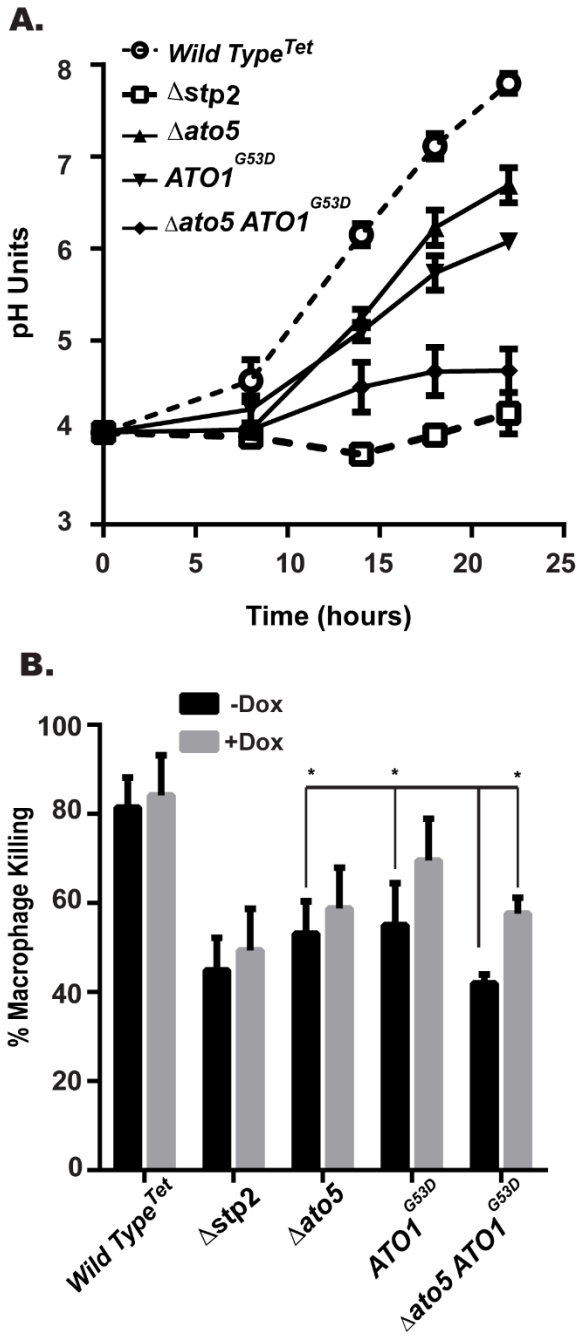

Figure 3-15. Double *ato* mutations results in additive alkalinization defects.

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Figure 3-15. Double *ato* mutations results in additive alkalinization defects. A) Wild-type (SC5314), *stp2*Δ (SVC17), *ato5*Δ^{Tet} (HDC45), and *ato5*Δ *ATO1*^{G53D} (HDC49) strains were incubated in YNB-CAA with an initial pH of 4.0 in aerated conditions at 37°C without Dox. pH of the cultures was taken at the indicated time points. B) Indicated strains were co-cultured with RAW264.7 macrophages in the presence and absence of 50 μg/mL of Dox in RPMI tissue culture media. Macrophage death was assessed based upon lactate dehydrogenase (LDH) release. An asterisk indicates a significant difference ($p<0.01$) relative to the double *ATO1*^{G53D} *ato5*Δ strain grown in the absence of Dox.

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Discussion

We show here that members of the *ATO* gene family are important mediators of the ability of *C. albicans* to neutralize its environment, both *in vitro* and in the macrophage phagolysosome. This conclusion is supported by evidence that individual mutants in *ATO1* (*ATO1*^{G53D}) or *ATO5 (ato5*Δ*)*, raise extracellular pH more slowly during *in vitro* growth in amino acid-rich conditions (Figure 3-4), release less ammonia (Figure 3-5), and are slower to germinate (Figure 3-6). In contact with macrophages, *ato* mutants occupy an acidic phagolysosome (Figure 3-10), which reduces hyphal growth and fungal survival while increasing macrophage integrity (Figure 3-9). While it is reasonable to infer that the defect in germination is directly responsible for the reduction in macrophage damage, recent publications attribute some of this damage to fungal-dependent induction of pyroptosis (156, 157). It is possible that the aberrant maturation of the *C. albicans*-containing phagosome resulting from the failure to acidify might reduce pyroptosis and thus also contribute to improved macrophage survival and, indeed, we have evidence that this is the case (S. Vylkova, H. Danhof, and M. Lorenz, unpublished observations).

The phenotype of a *ATO1*^{G53D} *ato5*Δ double mutant is additive and are similar to the previously reported *stp2*Δ strain (Figure 3-15) (94, 97). *ATO1* and *ATO5* are both transcriptionally regulated by Stp2p, though this could be direct or indirect (Figure 3-12). Further, overexpression of multiple *ATO* genes enhances *in vitro* alkalinization (Figure 3-14), suggesting that there is a broad role for this family in the pH alteration phenomenon.

Transcript profiling established that many *ATO* genes are significantly up-regulated during environmental alkalinization and an overlapping, but not identical, set is

also up-regulated during phagocytosis (87, 94). We show here that the induction of at least some *ATO* genes is largely or entirely Stp2p-dependent, emphasizing the central role of this transcription factor in regulating the metabolic changes that support the fitness of *C. albicans* in contact with phagocytic cells. The dramatic expansion of this gene family strongly suggests that differential functions and/or regulation exist between them, given the potential for redundancy. Indeed, we found this to be the case as overexpression analysis showed that many, but not all, *ATO* genes promote environmental alkalinization in the conditions tested (Figure 3-14).

We attempted to suppress the phenotypes of the *stp2*Δ mutant by constitutively expressing individual *ATO* genes (Figure 3-13). We were surprised to find that these overexpression strains failed to grow on media in which amino acids were the sole carbon source, though they were viable when glucose was present, which suppressed alkalinization. There is evidence for homo-multimeric and hetero-multimeric interactions between Ato homologs in yeast (103, 104) and our data would support the idea that these proteins form one or more functional complexes. In the *stp2*Δ mutant, either the correct stoichiometry is not maintained or a key non-*ATO* target of Stp2p is missing. Elucidating the details of the potentially complex functions of the *C. albicans* Ato molecular machine will require further study and will be elaborated on in Chapter 6.

Chapter Four:

Candida albicans has multiple mechanisms to raise extracellular pH

Introduction

To survive within the diverse range of niches of the human host, *Candida albicans* must rapidly sense and respond to the microenvironment. As a long term resident within a human host *C. albicans* must survive a wide range of environmental conditions including ambient pH as low as 2 or as high as 10 depending on anatomical niche. These variations in pH can impact cell physiology such as membrane potential, protein function, and micronutrient transport. Therefore, the mechanisms of response to extracellular pH signals are important to fungal survival and virulence.

Evolution of tolerance to distinct pH as seen in gene families optimized to work at different pH is provided by secreted aspartyl protease family (*SAPs).* As discussed in the introduction, the Sap enzymes are encoded by a 10-member gene family exhibits substantially different expression patterns, preferred cleavage sites, and optimal pH conditions that facilitate diverse functionality in distinct physiological conditions (77). Sap1-3 have the highest activity at very low pH and are expressed during phenotypic switching from white to opaque cells (80). Conversely, expression of *SAP4-SAP6* is the highest at neutral pH and co-regulated by the transcription factor *TEC1* with the morphological switch from yeast to hyphal form (81). Taken together, the diversity of the Sap proteins facilitates *C. albicans* survival and pathogenicity within the host through nutrient acquisition, host tissue adhesion and invasion, and modulation of interactions with immune cells which is a remarkable range of function for a highly homologous single gene family.

Another example of the importance of pH adaptation in *C. albicans* within divergent host niches is evident in the homologous glycosidases, Phr1 and Phr2, which are inversely regulated by ambient pH (158, 159). *PHR1* is necessary for growth at pH less than 5 and *phr1*Δ are mutants are avirulent in mouse model of infection with low pH, such as vaginitis, but are fully virulent in neutral infection sites such as bloodstream disseminated infection models (159, 160). Conversely, *PHR1,* which is transcriptionally controlled by Rim101, is necessary for virulence in bloodstream infections but is dispensable for vaginal infections (159, 161, 162).

Responses to alkaline pH have been studied in multiple fungal species and are critical for colonization and pathogenicity. Signal cascades in response to ambient pH in fungi were first reported in *S. cerevisiae* and *A. nidulans* in the now extensively studied PalC/Rim101 pathways (163, 164). The Rim101 pathway is conserved in *C. albicans* where alkaline ambient pH is sensed at the cell membrane by Rim31 and proteolytic cleavage activates the transcription factor Rim101 responsible for upregulation of gene expression (165). This signalling pathway is necessary for induction of hyphal formation in response to neutral pH and full virulence of *C. albicans (161, 166).*

In contrast, adaptation responses to low pH in fungi are less well understood than adaptation to neutral pH, although both are critical for colonization of the host. Two transcription factors have been identified in *C. albicans* to be responsive to acidic conditions. *MNL1* has been shown to mediate weak acid stress response through activation of genes necessary for acid tolerance (167). *WAR1* induces expression of the carboxylic acid exporter *PDR12 (168).* This activation has been shown to be important

for resistance to sorbate stress. However, neither *MNL1* nor *WAR1* has been fully characterized and therefore our understanding of cellular responses to acidic conditions is limited.

This chapter presents work that began to further test our model of alkalinization where the extruded ammonia is generated from the deamination of amino acids (Figure 1-1) (94). However, during the course of the experiments presented here it was elucidated that deletion of *STP2* results in only mild attenuation of virulence in whole animal models (97), and mutants in components of the SPS system are fully virulent (98) despite the apparent reduction of expression in many key components of amino acid utilization. These findings suggested that perhaps other mechanisms of alkalinization and acid tolerance were utilized *in vivo.*

Taken together, this evidence prompted us to generate a new hypothesis that *C. albicans* possesses additional mechanisms to alter the extracellular pH. Weak organic acids can be metabolized by *C. albicans* and have been shown to impact cell wall composition and immune cell recognition (115). These acids are generated as metabolic byproducts of both fungi and bacteria, which suggests they present both risk of weak acid stress and reward of energy generation to *C. albicans*. The experiments presented in this chapter are my contribution to a collaborative project in our lab where we have uncovered another mechanism to raise extracellular pH by *C. albicans* that is genetically distinct from amino acid catabolism and is independent of ammonia release.

Results

Candida albicans utilization of carboxylic acids raises extracellular pH

In order to test our working model that ammonia released from cells catabolizing amino acids is derived from the deamination of amino acids (Figure 1-1), liquid alkalinization assays were performed in cultures containing glutamate or serine as compared to the cognate deaminated molecules α-ketoglutarate (aKG) or pyruvate, respectively. The prediction was that cultures containing amino acids would raise the extracellular pH and that utilization of deaminated compounds would not result in pH change. Contrary to our expectation, we found that cultures containing aKG or glutamate as a sole carbon source rapidly raised the pH of the medium (Figure 4-1B), despite growing more slowly than casamino acid or glucose control cultures (Figure 4-1A). Further experiments revealed that utilization of other carboxylic acids, acetate and lactate, also supported moderate growth (Figure 4-1C) and significant pH change (Figure 4-1D).

These results led us to investigate whether the observed neutralization correlated with extrusion of ammonia from the cells. Ammonia release assays of wild-type (SC5314) cells were performed as described previously utilizing casamino acids, glutamate, serine, α-ketoglutarate, acetate or lactate as a sole carbon source. Consistent with previous results, cells utilizing casamino acids extruded significant amounts of ammonia, likewise the individual amino acids glutamate and serine produced ammonia release; however, utilization of pyruvate, α-ketoglutarate, acetate, and lactate did not result in ammonia (Figure 4-2). This suggested that alkalinization observed during amino

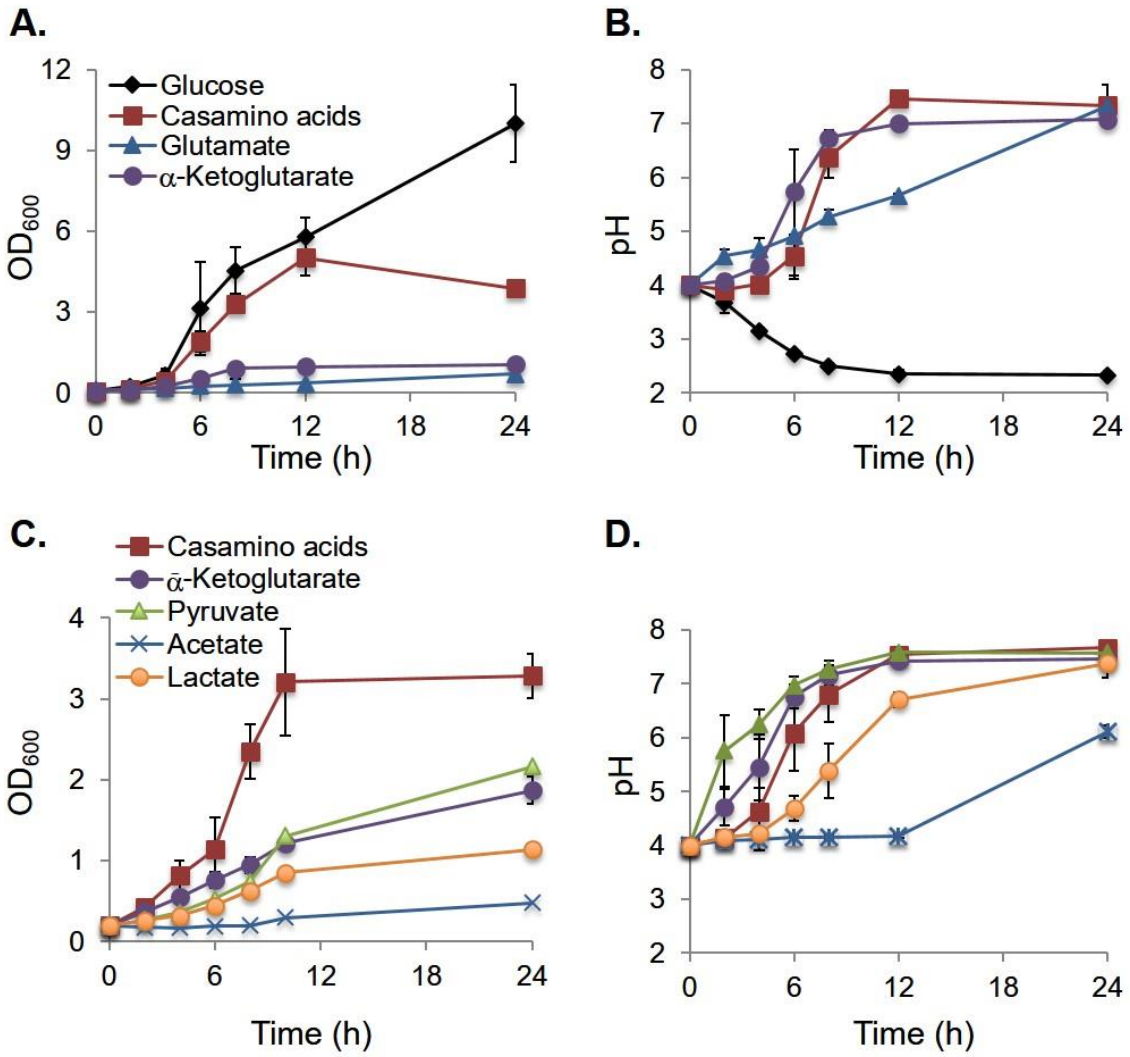

Figure 4-1. Carboxylic acids support robust extracellular alkalinization. Wild-type (SC5314) cells were grown in minimal YNB medium containing 10mM of the indicated compound as the sole carbon source at 37°C for 24 hours, monitoring growth (by optical density; A, C) and the pH of the culture media (using a pH probe; B, D). (A) Growth of *C. albicans* in glucose, casamino acids, glutamate, or a-ketoglutarate. (B) pH of the cultures in panel A. (C) Other carboxylic acids also support neutralization, including pyruvate, acetate and lactate. (D) pH of the cultures in panel C.

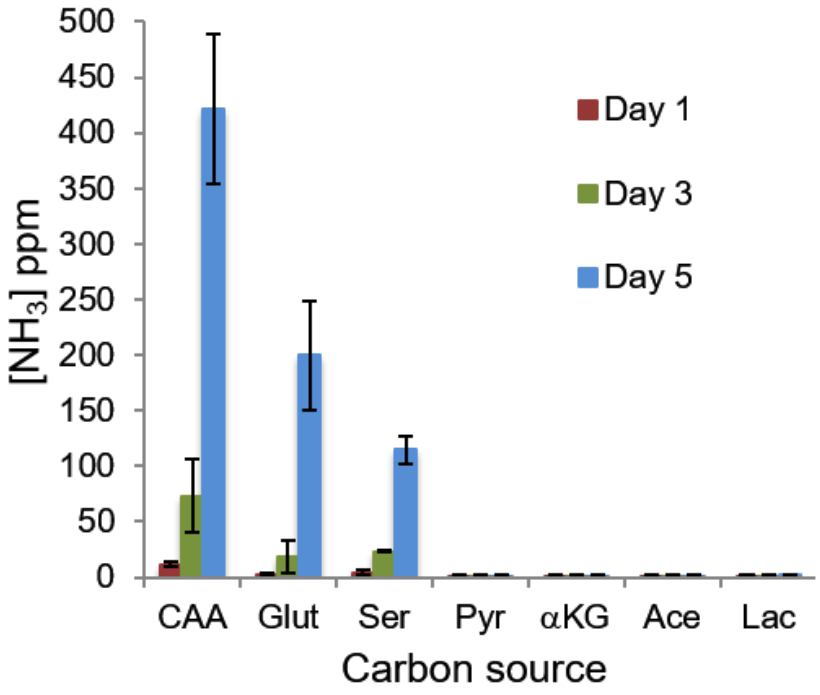

Figure 4-2. Media neutralization induced by growth on carboxylic acids does not generate ammonia. Wild-type (SC5314) cells were spotted onto solid YNB media with the indicated compound as the sole carbon source and allowed to develop into a colony at 37°C. Directly apposed to the colony, a small reservoir was affixed to the lid of the petri dish and filled with 10% citric acid. At the indicated times, a sample of the liquid in the acid trap was removed and assayed for nitrogen content using the Nessler's reagent, which is expressed as parts per million (ppm). CAA, casamino acids; Glut, glutamate; Ser; serine; Pyr, pyruvate; aKG, a-ketoglutarate; Ace, acetate; Lac, lactate.

acid utilization was distinct from the alkalinization observed during carboxylic acid utilization.

Mutants that our group has established as having defects in alkalinization during amino acid catabolism as in Chapter 3 were tested for alkalinization during α-ketoglutarate utilization. As previously described, *stp2Δ* cells fail to alkalinize during amino acid catabolism (Figure 4-3 B and D)*;* additionally, *ato5Δ, ATO1*^{G53D}, and *ach1Δ* strains have a significant delay in alkalinization at early time points compared to wild-type (SC5314) controls (Figure 4-3 B and D). However, all four mutant strains were able to grow and alkalinize as robustly as wild-type in α-ketoglutarate medium as a sole carbon source (Figure 4-3 E and F). These results strongly support the hypothesis that environmental alkalinization utilizing amino acids and α-ketoglutarate are distinct physiological processes in *C. albicans.*

Since the strains with established alkalinization defects during amino acid utilization had no such defects when growing on α-ketoglutarate, we next performed screens in liquid media of several available mutant libraries to identify genes that are important for alkalinization on carboxylic acids. All primary candidates were validated through a secondary screening to eliminate false negative candidates due to poor growth. One candidate gene was found, the transcription factor *CWT1*. This transcription factor has been characterized to be required for proper cell wall organization (169) and shows reduced ability to alkalinize the media when utilizing α-ketoglutarate as a sole carbon source (Slavena Vylkova, personal communication).

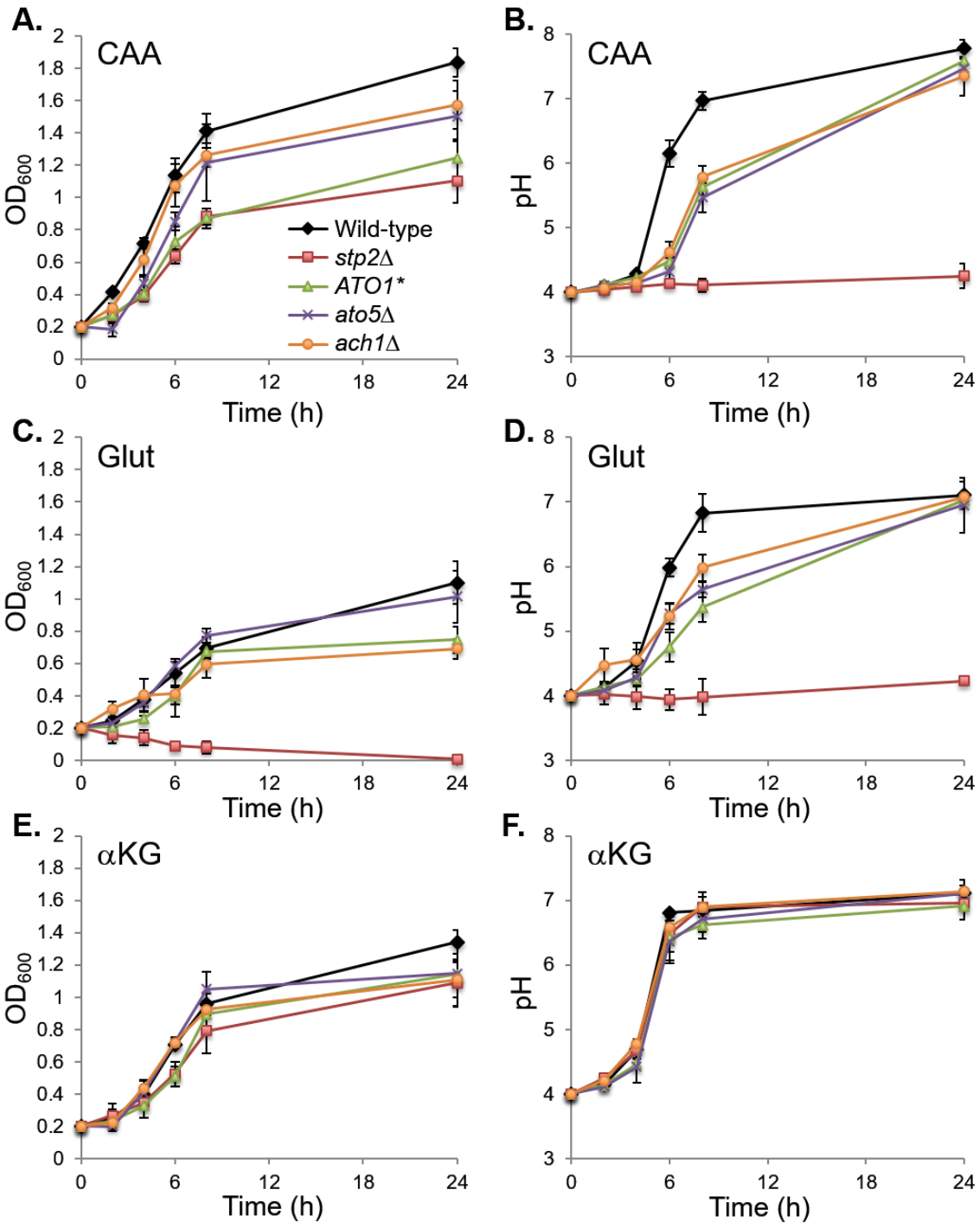

Figure 4-3. Amino acid- and carboxylic acid-driven alkalinization are genetically distinct.

Figure 4-3. Amino acid- and carboxylic acid-driven alkalinization are genetically distinct. The wild-type (SC5314), *stp2Δ* (SVC17), *ato5Δ* (HDC17), *ATO1*^{G53D} (Can572), and *ach1Δ* (Can200) were grown in minimal liquid YNB media with the indicated carbon source, casamino acids (CAA: A, B); glutamate (Glut; C, D); or a-ketoglutarate (aKG; E, F). Culture density (A, C, E) and culture pH (B, D, F) were measured at the indicated times. Genotypes are homozygous except for the *ATO1*^{G53D} allele, which is expressed from the *ACT1* promoter in the parent of the other mutants (SC5314). Results are expressed as means +/- standard deviation of triplicate experiments.

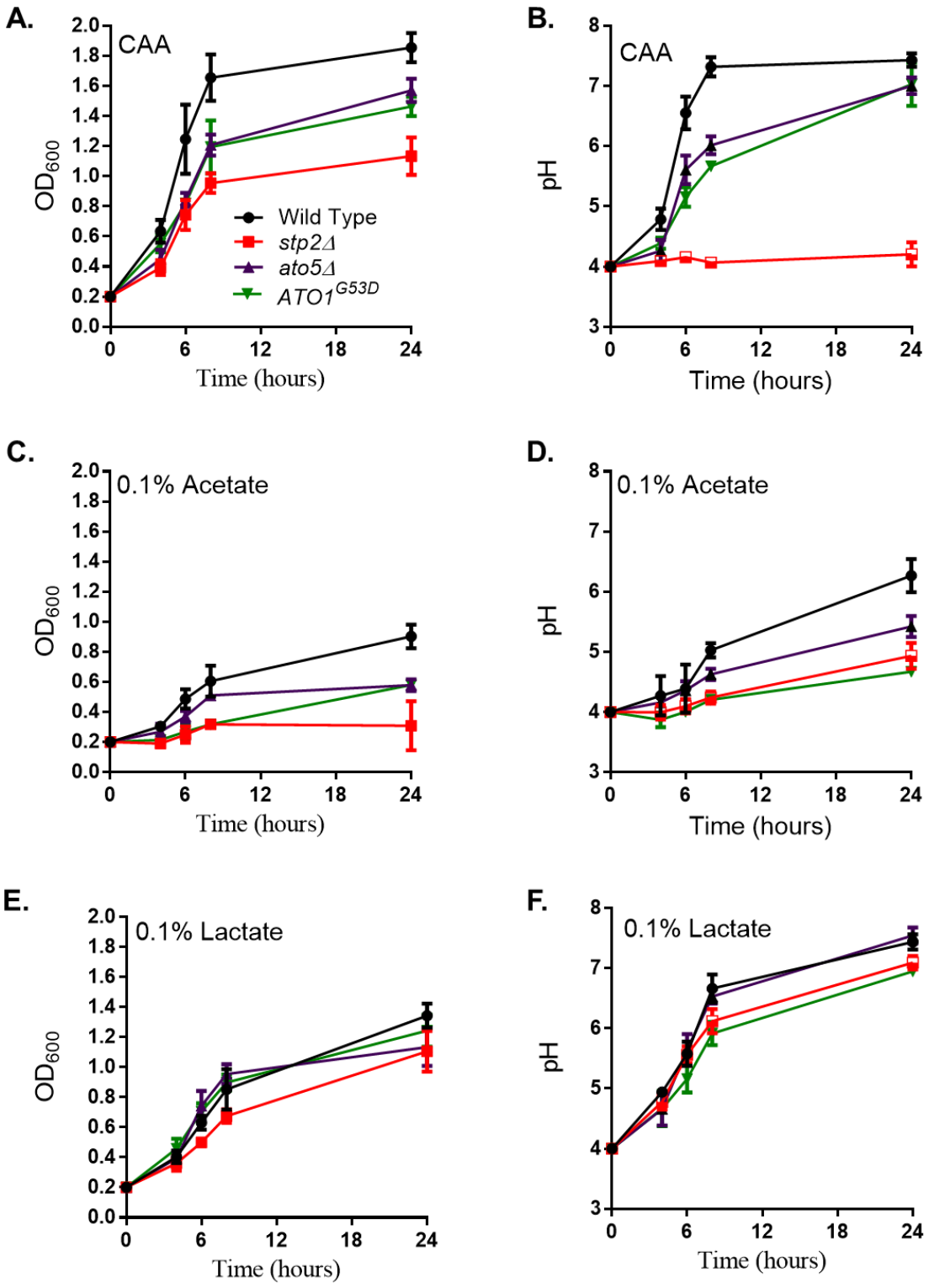

Figure 4-4. Carboxylic acids in addition to casamino acids alter alkalinization.

Figure 4-4. Carboxylic acids in addition to casamino acids alter alkalinization.

The wild-type (SC5314), *stp2Δ* (SVC17), *ato5Δ* (HDC17), and *ATO1*^{G53D} (Can572) were grown in minimal liquid YNB media with the indicated carbon source, casamino acids only (CAA: A, B); casamino acids + 0.1% Acetate (0.1% Acetate; C, D); or casamino acids + 0.1% lactate (0.1% lactate; E, F). Culture density (A, C, E) and culture pH (B, D, F) were measured at the indicated times. Genotypes are homozygous except for the *ATO1*^{G53D} allele, which is expressed from the *ACT1* promoter in the parent of the other mutants (SC5314). Results are expressed as means +/- standard deviation of triplicate experiments.

In order to elucidate if the presence of carboxylic acids could suppress the alkalinization defects of mutant strains that have alkalinization defects during amino acid catabolism, cells grown in casamino acid media (CAA) were also assayed in medium containing CAA + a carboxylic acid (acetate or lactate; pH 4). To our surprise, mutant cells grown in CAA + lactate grew and alkalinized at wild-type levels (Figure 4-4 E and F) as compared to CAA only medium (Figure 4-4 A and B). Conversely, the addition of 0.1% acetate to CAA medium slowed growth of *ATO1*^{G53D} cells and exacerbated the alkalinization defect to near the levels of cells lacking *STP2* (Figure 4-4 C and D). Taken together these data suggest that the degree of alkalinization depends upon the carboxylic acid present in the media; and that *ato1*Δ, *ATO1*^{G53D} as well as *stp2Δ,* mutants are more sensitive to the presence of acetate than other carboxylic acids. This result is significant as it is the first evidence of acetate toxicity observed in *C. albicans ato* mutant strains and may provide a potential link to acetate transport, which will be discussed in Chapter 5.

Neutral pH alone is not sufficient to induce hyphal morphogenesis

As mentioned in the introduction, this project was a collaborative effort among members of our lab and Elisa Vesely investigated the morphology of cells that were grown in YNB + 2% (w/v) casamino acids, α-ketoglutarate, pyruvate, acetate, and lactate and grown at 37°C over a period of 24 hours. At six, eight, and 24 hours cells were removed from the culture medium, fixed in paraformaldehyde, and visualized by DIC microscopy. Only cells that were grown in casamino acids medium formed filaments despite all samples reaching near neutral by eight hours (Elisa Vesely, personal communication). These data further support the hypothesis that environmental

alkalinization as a result of amino acid utilization is distinct from alkalinization during carboxylic acid utilization.

In order to determine if the presence of amino acids alone is sufficient to induce hyphal morphogenesis, strains were grown in liquid culture with 20 mM α-ketoglutarate or glutamate as a sole carbon source or in combination at 37°C and analyzed for hyphal formation. Representative images are shown in Figure 4-5 of the 10-hour time point where the pH of all cultures are similar. Germination has occurred in cells grown in glutamate alone or α-ketoglutarate + glutamate but not in cultures that contained α-ketoglutarate as a sole carbon source which is consistent with my colleague Elisa Veseley's observations of cells grown in α-ketoglutarate medium remain in yeast form. Taken together, these data suggest that utilization of amino acids provides a signal for hyphal morphogenesis that is not present when cells are utilizing α-ketoglutarate alone.

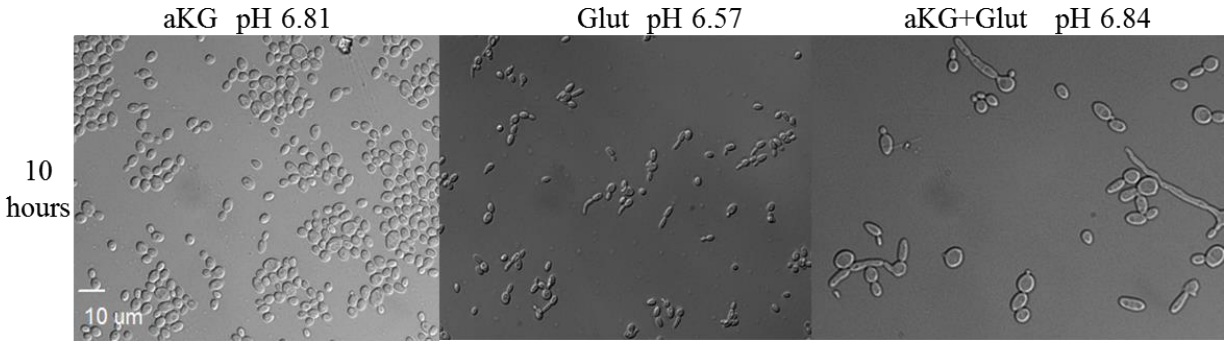

Figure 4-5. Glutamate presence induces hyphal formation that is absent in carboxylic acids alone. Cells of the wild-type SC5314 strain were grown overnight in YPD, then washed and diluted into YNB + 20 mM of the indicated compound present as the carbon source and grown at 37°C for ten hours prior to fixing and imaging. aKG, α-ketoglutarate; Glut, glutamate. The scale bar in the lower left 10µm.

Discussion

In previous studies our group has established that during amino acid catabolism ammonia is released from the cell which promotes hyphal production; cells that have defects in the ability to raise the pH also have reduced ability to form hyphae (94, 97, 129). Experiments presented in this chapter began with the hypothesis that neutralization of the extracellular environment was dependent upon ammonia release. The results clearly demonstrate that environmental alkalinization can occur by at least two genetically distinct cellular responses in *C. albicans*: one that involves utilization of carboxylic acids such as α-ketoglutarate, acetate, and lactate (Figure 4-1); and one that is the result of amino acid catabolism.

Amino acid catabolism results in the extrusion of ammonia that is absent in culture medium containing only carboxylic acids as a sole carbon source (Figure 4-2). These data present the first evidence of a clear distinction between the two alkalization mechanisms. Screens of available mutant libraries revealed that loss of the transcription factor *CWT1* impaired alkalinization on α-ketoglutarate medium but not medium containing casamino acids (Slavena Vylkova, personal communication). Additional support for distinct alkalinization responses was presented in Figure 4-3 where none of the established mutant strains that are defective in alkalinization on medium with amino acids were impaired in alkalinization while utilizing α-ketoglutarate.

When these same mutant strains were tested on combinations of casamino acid and the carboxylic acids acetate and lactate in liquid cultures at pH 4, it was observed that growth and alkalinization was impaired in the presence of acetate (Figure 4-4 C and D)

but not in the presence of lactate (Figure 4-4 E and F). These results suggest that even at low levels acetate is toxic to *ato* mutant strains, which has never been observed before when tested on solid growth medium. RNA-seq analysis revealed *ATO* gene expression is broadly controlled by *STP2*, so *stp2Δ* mutants would have impaired expression of many *ATO* genes potentially impacting acetate sensitivity (M. Lorenz, S. Vylkova, and H. Danhof unpublished observations). These results present a possible link between the alkalinization phenotypes of observed in *C. albicans* discussed in Chapter 3 and the acetate phenotypes discussed in Chapter 5, and therefore will be more fully discussed in the perspectives chapter.

When a single amino acid, glutamate, is utilized as a carbon source ammonia release is observed (Figure 4-2) and when glutamate is added to α-ketoglutarate medium germination is observed (Figure 4-5). However, while utilization of carboxylic acids rapidly raised the extracellular pH, no morphogenic switch to filamentous form occurred unless amino acids are present in the culture medium (Elisa Vesely, personal communication and Figure 4-5) strongly suggests that in these *in vitro* assays neutral pH alone is not sufficient to induce germination. Taken together, these data suggest that the presence of ammonia is critical to the morphogenic switch. However, it remains to be elucidated if the combination of the media acts as an inducer of hyphal morphogenesis directly, or perhaps overcomes some sort of repression of filamentation that is exerted by the presence of carboxylic acids. There is some reported evidence that the presence of extracellular methionine and proline promotes hyphal morphogenesis (50), however this is the first report of glutamate affecting filamentation.

Overall, data presented in this chapter provide strong evidence that *C. albicans* possesses multiple mechanisms to achieve extracellular neutralization, which is logical given the importance of pH modulation within various host niches. Additionally, induction of hyphal morphogenesis varies depending on carbon source, an intriguing finding that has broad implications on cell physiology of *C. albicans* which will be further explored in the perspectives chapter.

Chapter Five:

Ato proteins mediate cytosolic pH and amino acid utilization in *Saccharomyces cerevisiae*

Introduction

Transport mechanisms are fundamental to nutrient acquisition and despite being critically important to cellular metabolism are often overlooked. The ability to utilize non-preferred carbon sources, such carboxylic acids, is critical to fungal survival and yet presents complex challenges to cellular homeostasis. When pH of the extracellular environment is low, then weak acids are uncharged and can freely diffuse across the cell membrane. However, once in the neutral cytosolic environment deprotonation occurs, acidifying the cytoplasm and disrupting cellular redox potential and protein homeostasis. Deprotonation also traps the anion in the cytosol necessitating active transport out of the cell if not metabolized. These considerations suggest that cells must employ sophisticated and rapid efflux mechanisms.

Ato proteins are widely characterized for function in acetate transport/utilization

Ato family proteins, although named for ammonia transport in *S. cerevisiae*, have previously been characterized as required for utilization and/or tolerance of acetate stress. Homologs in *S. cerevisiae* and *Aspergillus nidulans* have established roles in facilitating active uptake of acetate into the cell (107, 109)*;* and the *Escherichia coli* homolog, YaaH/SatP has recently been characterized as an inward acetate-succinate transporter (110). A dominant mutant of *GPR1* (an *ATO* homolog) confers sensitivity to acetate at low pH; this mutant has a substitution within the conserved FGGTLN motif in the cytosolic N-terminal domain (111-113). We have shown, as discussed in Chapter 3, that in *C. albicans* this substitution results in reduction of environmental alkalinization.

S. cerevisiae Ato1 was originally named Ady2, as strains lacking this protein had a sporulation defect (Accumulation of DYads). Further investigations reported this protein was involved in active transport of acetate into *S. cerevisiae* (107) and recently lactate transport was also reported (108). Previous studies in our lab determined *S. cerevisiae* strains expressing the conserved glycine to aspartate mutation displayed acetate sensitivity that was concentration and pH dependent (149). Further, cells lacking all three Ato proteins had sporulation defects when acetate but not glycerol was the carbon source (149). Standard sporulation media contains acetate as the carbon source, suggesting that the carbon source may have been the cause of observed Ady2 phenotype. Despite being characterized as inward transporters in fungal species due to lack of acetate utilization, direct biochemical evidence to support this is lacking.

Ato protein predicted structure and regulation

S. cerevisiae and *Y. lipolytica* Ato homologs have been localized to the plasma membrane (103, 104, 111) and one of the defining characteristics of this broad protein family is conserved hydrophobic regions indicative of an integral membrane protein (Figure 5-1). However, with the characteristic six transmembrane domains, they do not resemble any previously identified carboxylic acid transporters which are predicted to have 12 membrane spanning domains (107, 114, 170). Similarly, the Ato homologs are divergent from known ammonium permeases which have 10 – 12 transmembrane spanning domains (100, 171).

Work done by the Barth group on *Y. lipolytica* Gpr1 has elucidated that, in addition to previously reported transcriptional induction (111), Ato homologs are

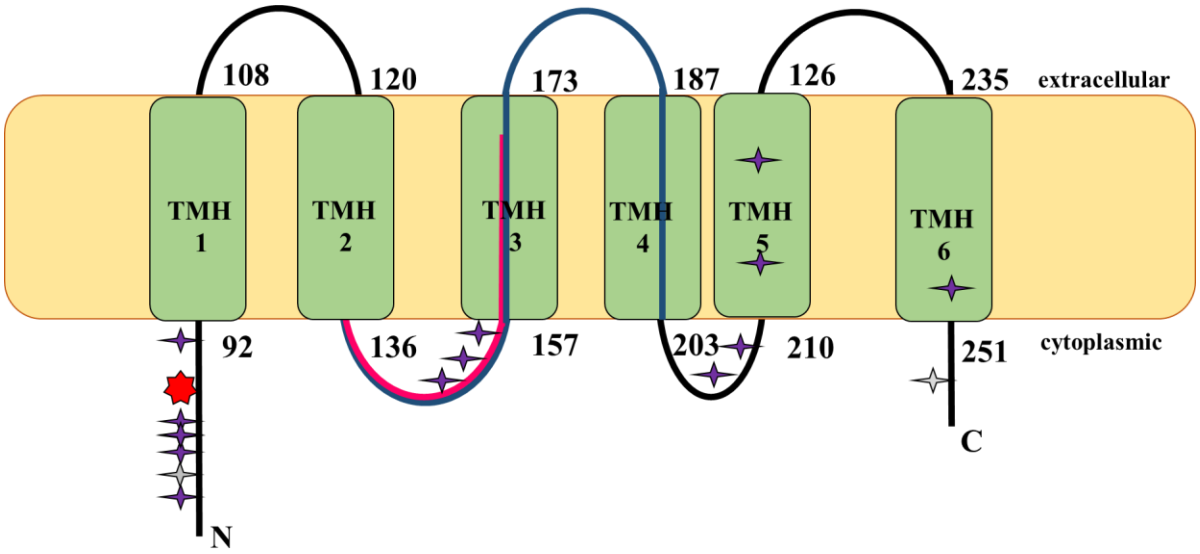

Figure 5-1. Schematic of the predicted Ato protein structure. TMHMM predicted topology of *S. cerevisiae* Ato1, the numbers indicate the position of the amino acids from the N- to C-termini. The six predicted transmembrane helices are depicted in green, with the putative ammonium transport signature highlighted in magenta. The blue highlighting indicates the voltage gated channel motif predicted by Phyre[2] analysis. Purple stars depict residues that when mutated confer acetate sensitivity, with the G72 dominant negative highlighted in red.

regulated at the post transcriptional level through phosphorylation events at serine-37 that is dependent upon the presence of acetate and the C-terminus of the protein (114). How this phosphorylation event occurs is unknown, however it validates the protein topology predictions that both the N- and the C-terminus are located in the cytosol. Additionally, Gpr1 has been suggested to exist as an oligomer with the potential to regulate acetate efflux (114), although the mechanism remains elusive. Similarly, as mentioned in the introduction recent work utilizing microscopy techniques has also suggested that *S. cerevisiae* Ato1 and Ato2 form homo-multimeric structures and that Ato1 forms a heterodimer with Ato2, however the physiologic importance of these observed associations remains unclear (103, 104).

The widespread conservation of the *ATO* family of genes as acetate transporters highlights their importance in utilization of alternative carbon sources. However, the data are not entirely consistent with these proteins operating solely as inward acetate transporters. Based upon previous studies in *S. cerevisiae*, *Y. lipolytica*, and our preliminary data I hypothesize that the Atos also transport acetate outward and contribute to the homeostasis of cytosolic pH. Work presented in this chapter aims to address Ato protein function in the maintenance of cytosolic pH when challenged with weak acid stress.

Results

S. cerevisiae Ato proteins self-associate

Structural and genetic evidence from many species strongly suggests that Ato proteins are acetate transporters. The dramatic expansion in *C. albicans* of the Ato protein family suggests that they are important virulence factors, and we have shown them to be linked to ammonia transport during alkalinization, which modulates interactions with the host immune system. It is tempting to speculate that *C. albicans* has adapted a conserved way of coping with cellular weak acid stresses such as the presence of acetate, to a new niche in which it faces acid stress, the macrophage phagolysosome; however, the mechanism of adaptation that could result in all the observed phenotypes remains unclear.

One possible way to achieve this diversity in Ato function is through hetero-multimeric complex formation with additional membrane proteins. Therefore, to test the hypothesis that the diverse range of Ato-associated phenotypes are a result of protein-protein interactions with other membrane transport proteins, I sought to analyze pairwise interactions with each of the three *S. cerevisiae* proteins utilizing a protein fragment complementation assay (132). This assay is in essence a yeast two-hybrid type experiment that has been optimized for characterization of membrane proteins. The protein complementation assay utilizes an *S. cerevisiae* strain that is lacking the endogenous *FCY1* gene, which encodes cytosine deaminase, an enzyme responsible for converting cytosine to uracil and allowing growth on media lacking uracil. In this system, *FCY1* has been split and one half is translationally fused behind the gene of interest on an inducible plasmid. The other half is fused to a target interaction partner on a separate inducible plasmid, and the two plasmids are co-expressed in a *fcy1*Δ strain.

Growth on media lacking uracil is indicative of reconstitution of Fcy1 enzymatic activity by close association of the proteins of interest and is therefore a readout of protein-protein interactions (132).

Interactions were observed between Ato proteins (Table 5-2) and results agreed with the previously reported Ato protein interactions which validated the assay (103, 104). Notably, the dominant negative mutations that reduce Ato1 function did not interrupt the protein-protein interactions (Table 5-2). Interactions between Ato proteins were confirmed by plasmid swapping. Next, we sought to identify potential interaction partners that were identified as biochemically or genetically linked to Atos and involved in nutrient or ion transport from publically available high throughput interaction screens and literature search (Table 5-1) (172, 173). None of the selected candidates interacted with any Ato protein in pair-wise fashion (Table 5-2).

S. cerevisiae strains lacking *ATO* genes are sensitive to acetate stress

Previous unpublished work suggested that *S. cerevisiae* strains over-expressing $ATO1^{G73D}$ or $ATO2^{G73D}$ are sensitive to acetate which was consistent with the characterization of *Y. lipolytica ATO* homolog *GPR1*. (114, 149). This sensitivity is increased at low pH where the influx of uncharged molecules increases leading to potential accumulation of anion in the cytosol (149). It has been reported by several studies that homologs of Ato proteins are involved in acetate uptake and utilization by fungal species, as discussed in the introduction (107, 109, 112). However, definitive biochemical validation of *S. cerevisiae* Atos solely as inward transporters has not been established. I hypothesized a novel function for the ATOs as acetate exporters crucial for maintaining cytosolic pH homeostasis during weak acid stresses. In order to test this

Table5-1. *S. cerevisiae* protein-protein interaction candidates chosen from primary screens (172, 173)					
Ato1-		**Ato2-**		**Ato3-**	
Protein	**Function**	**Protein**	**Function**	**Protein**	**Function**
Ato1	Acetate/Ammonia transport	**Ato1**	Acetate/Ammonia transport	**Ato1**	Acetate/Ammonia transport
Ato1^{G72D}	Acetate/Ammonia transport	**Ato1^{G72D}**	Acetate/Ammonia transport	**Ato2**	Acetate/Ammonia transport
Ato2^{G72D}	Acetate/Ammonia transport	**Ato2**	Acetate/Ammonia transport	**Ato3**	Acetate/Ammonia transport
Jen1	Acetate transport	**Ato2^{G72D}**	Acetate/Ammonia transport	**Rvs16**	Nutrient starvation response
Gef1	Chloride channel	**Ort1**	Ornithine transport	**Tpo3**	Polyamine transporter
Can1	Arginine permease	**Vma1**	Vacuolar ATPase		
Agp1	Amino acid transporter	**Cwh4**	Cell wall synthesis and glycosylation		
Gnp1	Amino acid transporter				

Table 5-2. Results of protein-protein interaction studies			
	Ato1	**Ato2**	**Ato3**
Ato1	+	+	-
Ato1^{G72D}	+	+	-
Ato2	+	+	-
Ato2^{G72D}	+	+	-
Ato3	-	-	+
Jen1	-	-	-
Gef1	-	-	-
Can1	-	-	-
Agp1	-	-	-
Gnp1	-	-	-
Ort1	-	-	-
Vma1	-	-	-
Cwh4	-	-	-
Rvs16	-	-	-
Tpo3	-	-	-

hypothesis, I performed liquid acetate toxicity assays where wild-type (Σ1278b) or strainslacking all three *ATO* genes (*ato* Δ/Δ/Δ) were grown in rich growth medium supplemented with acetate at low pH for one hour and then plated for colony forming units (Figure 5-2). *ato* Δ/Δ/Δ strains were hypersensitive to the presence of acetate in a time and concentration dependent manner (Figure 5-3 A and B).

The rapid loss of viability of *ato* Δ/Δ/Δ cells suggested cellular acetate toxicity rather than disruption in active uptake, as the low pH of the culture medium allows free diffusion across the cell membrane. Therefore, we hypothesized that acidification of the cytosol due to an inability to efflux acetate was the cause for the loss of viability in *ato* Δ/Δ/Δ mutants. To test the impact of acetate stress to cytosolic pH homeostasis we utilized pHluorin, a pH sensitive derivative of GFP which loses signal intensity at low pH (174, 175), to approximate intracellular pH. Because the potential changes in the cytosolic pH were anticipated to be both rapid and possibly transient, wild-type (Σ1278b) or *ato* Δ/Δ/Δ cells were analyzed via fluorescence microscopy in real time by changing media conditions of cells in a microfluidics chamber.

In agreement with our hypothesis, the cytosolic pH of *ato* Δ/Δ/Δ was strains greatly reduced almost immediately upon addition of acetate to the media compared to wild-type controls (Figure 5-4). While some pHluorin aggregation was seen in wild-type cells as a potential indicator of stress during the 7-minute acetate challenge, no overall loss of GFP signal was observed. Further, wild-type cells resolved the aggregates quickly and maintained fluorescence upon removal of acetate from the media, while the *ato* Δ/Δ/Δ strain did not recover fluorescence even if the experiment was carried out to 30 minutes (Figure 5-4 and data not shown).

Defects in cytosolic pH maintenance are not due to the presence of glucose

As mentioned in the introduction, in the presence of glucose *S. cerevisiae* utilizes catabolite repression to downregulate expression of alternative carbon metabolism genes, which should impact expression of the *ATO* genes as well as many other genes in the cell (reviewed in (83)). To validate that the observed reduction in cytosolic pH in the above experiment was due to the loss of Ato proteins and not an additional factor that was repressed in the culture conditions, strains were incubated in medium containing casamino acids and glycerol to relieve glucose repression for three hours prior to visualization by microscopy as described above. GFP fluorescence was decreased in the strain lacking *ATO* genes with similar kinetics as were observed in SD-ura+glucose conditions (Figure 5-5) indicating that the loss of GFP signal intensity is due to the absence of Ato proteins and not some off target effect due to the assay conditions.

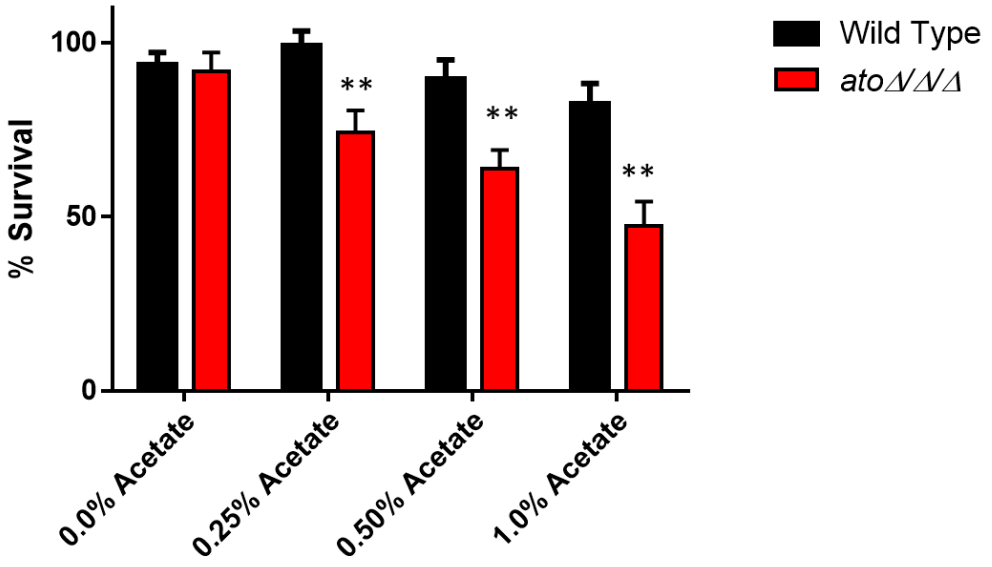

Figure 5-2. *S. cerevisiae* strains lacking *ATO* genes are sensitive to acetate. Wild-type (σ 1278b) and *ato* Δ/Δ/Δ (LY342) were incubated in SD-uracil pH4 with the addition of increasing amounts of acetate for one hour and plated for colony forming units (CFU) on rich media (YPD). Results are reported as means +/- the standard deviation of triplicate experiments. (** p-value <0.001)

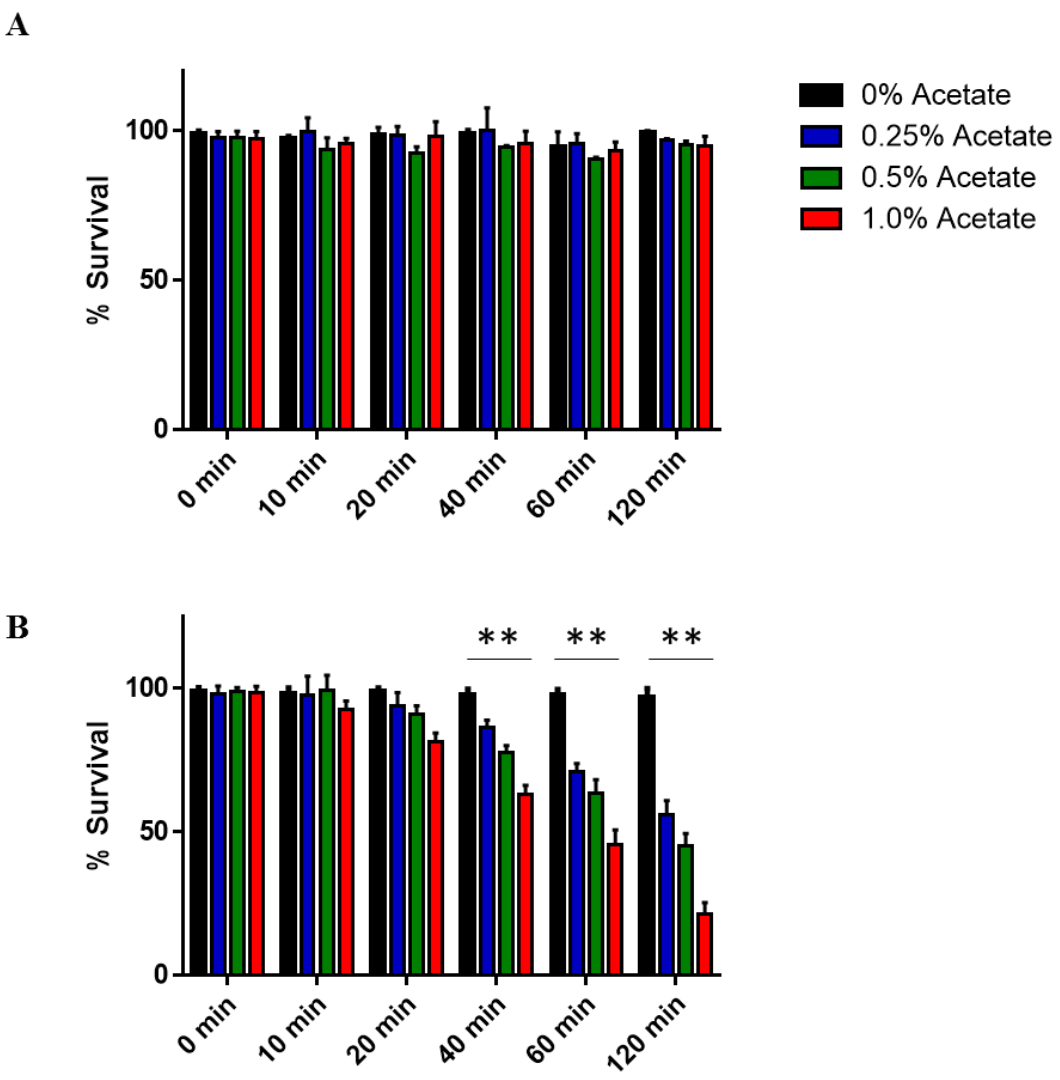

Figure 5-3. *S. cerevisiae* acetate sensitivity is time and concentration dependent. Wild-type (σ 1278b) (A) and *ato* Δ/Δ/Δ (LY342) (B) were incubated in SD-ura pH4 with the addition of increasing amounts of acetate and plated for CFUs on rich media (YPD) at the indicated time points. Results are reported as means +/- the standard deviation of triplicate experiments (** P-value < 0.001).

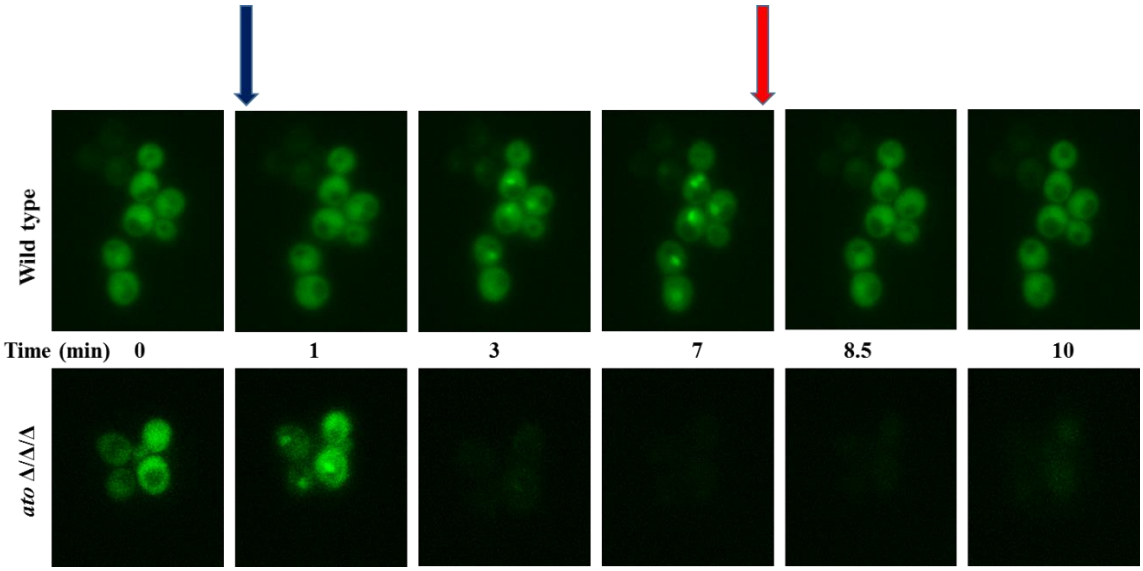

Figure 5-4. *ato* Δ/Δ/Δ mutants are unable to maintain cytosolic pH during acetate stress. Wild-type (σ 1278b) *ato* Δ/Δ/Δ (LY342) strains expressing pHluorin were placed in a Cellasic microfluidics chamber and SD-ura (pH 4) medium was flowed over the cells. Media was changed to SD-ura + 0.25% acetate (pH4) at the start of the experiment (Blue arrow) and returned to SD-ura (pH 4) medium after 7 minutes (red arrow). GFP fluorescence was monitored at 15 second intervals throughout the experiment. Representative images are presented of live cell imaging performed at least three times.

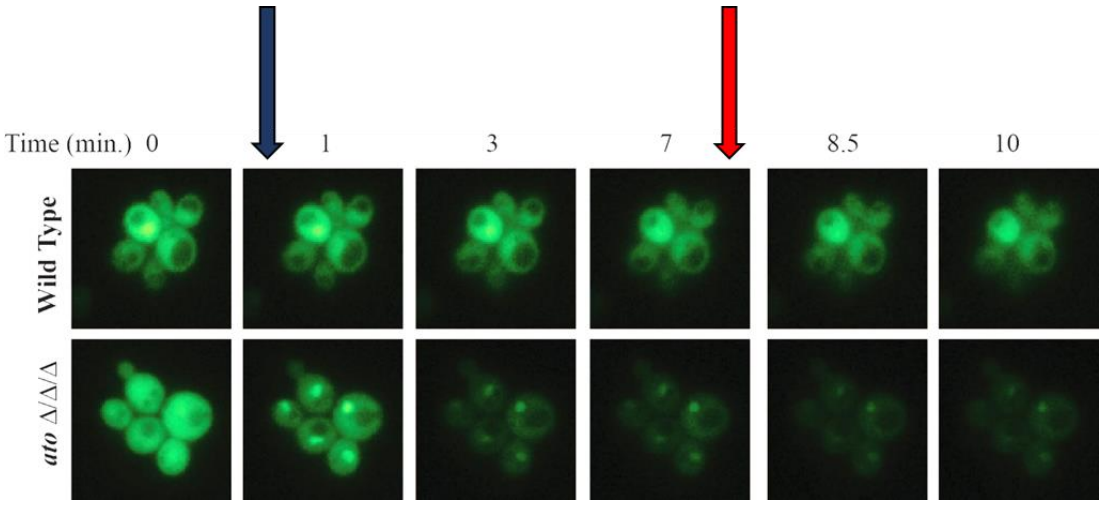

Figure 5-5. Glucose presence does not impact maintenance of cytosolic pH during acetate stress. Wild-type (Σ1278b) *ato* Δ/Δ/Δ (LY342) strains expressing pHluorin were placed in a Cellasic microfluidics chamber and SD -ura +glycerol (pH 4) medium was flowed over the cells. Media was changed to SD -ura + glycerol + 0.25% acetate (pH4) at the start of the experiment (Blue arrow) and returned to SD-ura (pH 4) medium after 7 minutes (red arrow). GFP fluorescence was monitored using at 15 second intervals throughout the experiment. Representative images are presented of live cell imaging performed at least three times.

ATO1 maintains cytosolic pH during acetate stress

In order to determine the contribution of the individual Ato proteins to acetate toxicity and cytosolic pH homeostasis strains lacking each Ato protein, as well as a strain expressing a dominant negative mutation in *ATO1 (ATO1^{G72D})* were grown in SD-ura (pH 4) where all strains grew well (Figure 5-6A). Wild-type (Σ1278b) and a strain lacking all *ATO* genes (*ato* Δ/Δ/Δ) were grown in SD-ura+ increasing amounts of acetate (0.1%, 0.25%, 0.5%, and 1%) and toxicity was observed at all concentrations above 0.25% which mildly impaired growth of the *ato* Δ/Δ/Δ strain (Figure 5-6B). Therefore, all strains as in (A) were grown in 0.25% acetate media and growth was observed over time. *ato1*Δ mutant strains had a substantial growth defect in the presence of acetate, while mutants in *ATO2* had a slight growth defect and *ato3*Δ mutants grew similarly to wild-type controls (Figure 5-6C). This suggests that *ATO1* is primarily responsible for the loss of cytosolic pH homeostasis observed in *ato* Δ/Δ/Δ cells.

To discern if the toxicity observed in *ato1*Δ mutants was a result of loss of regulated cytosolic pH, live cell microfluidics was performed as described previously. Rapid loss of fluorescence was observed in both the *ato* Δ/Δ/Δ and the *ato1Δ* cells while *ato2Δ* cells had a slightly diminished intensity and *ato3Δ* retained a strong signal throughout the experiment similarly to wild-type cells (Figure 5-7). Taken together, from these experiments we conclude that Ato1 is primarily responsible for cytosolic pH maintenance; Ato2 contributes to cytosolic pH maintenance in a lesser role than Ato1; and loss of Ato3 does not significantly impair pH homeostasis of the cell when challenged with acetate stress.

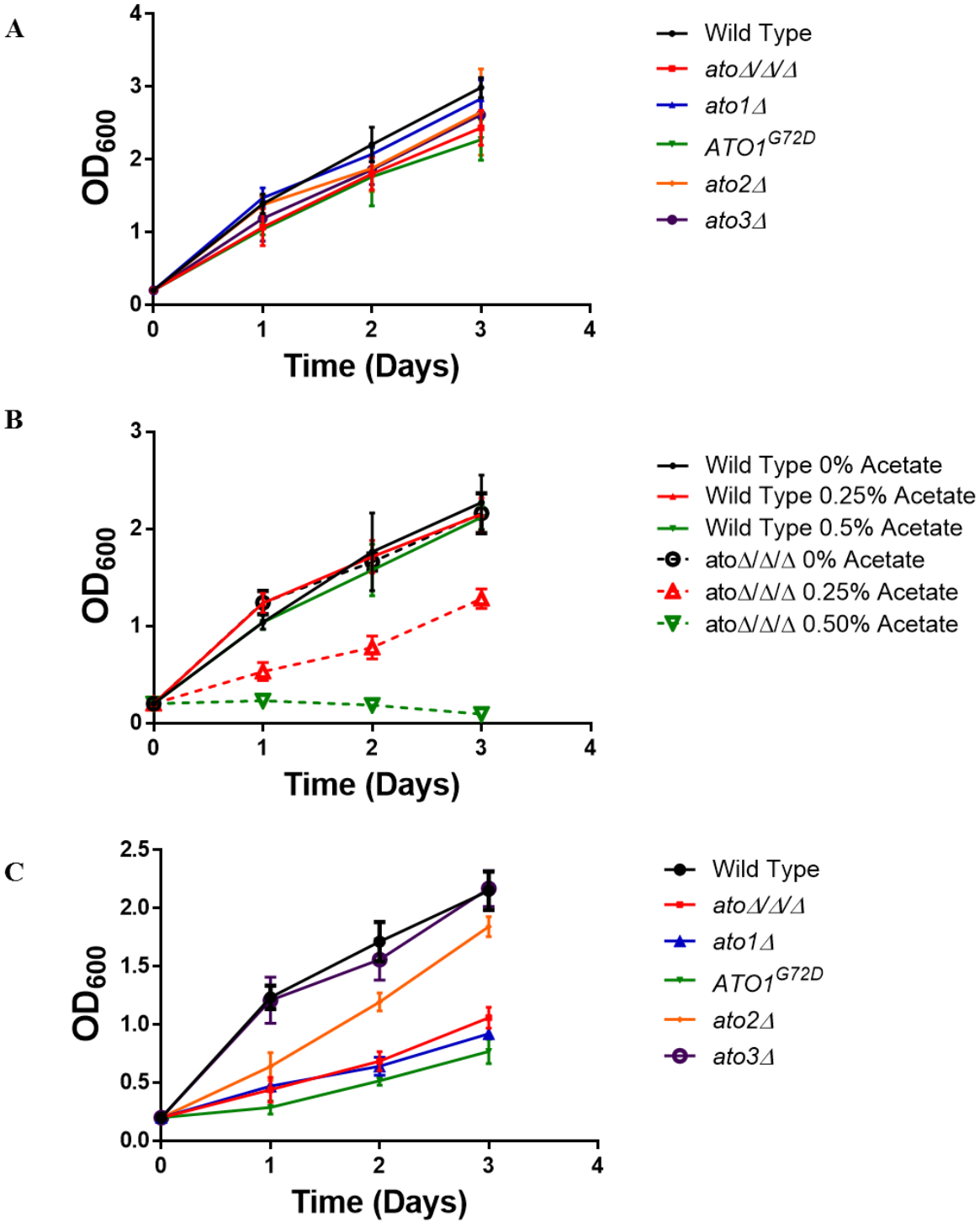

Figure 5-6. Disruption of Ato1 results in acetate sensitivity.

Figure 5-6. Disruption of Ato1 results in acetate sensitivity. Wild-type (Σ1278b), *ato* Δ/Δ/Δ (LY342), *ato1Δ* (LY338), *ATO1*^{G73D} (DY1), *ato2Δ* (LY351), and *ato3Δ* (LY355) were grown were incubated in SD-ura (pH 4) (B) Wild-type (Σ1278b) and a strain lacking all *ATO* genes (*ato* Δ/Δ/Δ) were incubated and in SD-ura +increasing amounts of acetate (0.0%, 0.25%, 0.5%,) (C) Indicated strains were incubated in 0.25% acetate media. Growth of the cells was measured by OD600 readings at the indicated time points. Results are reported as mean values +/- SD of triplicate assays.

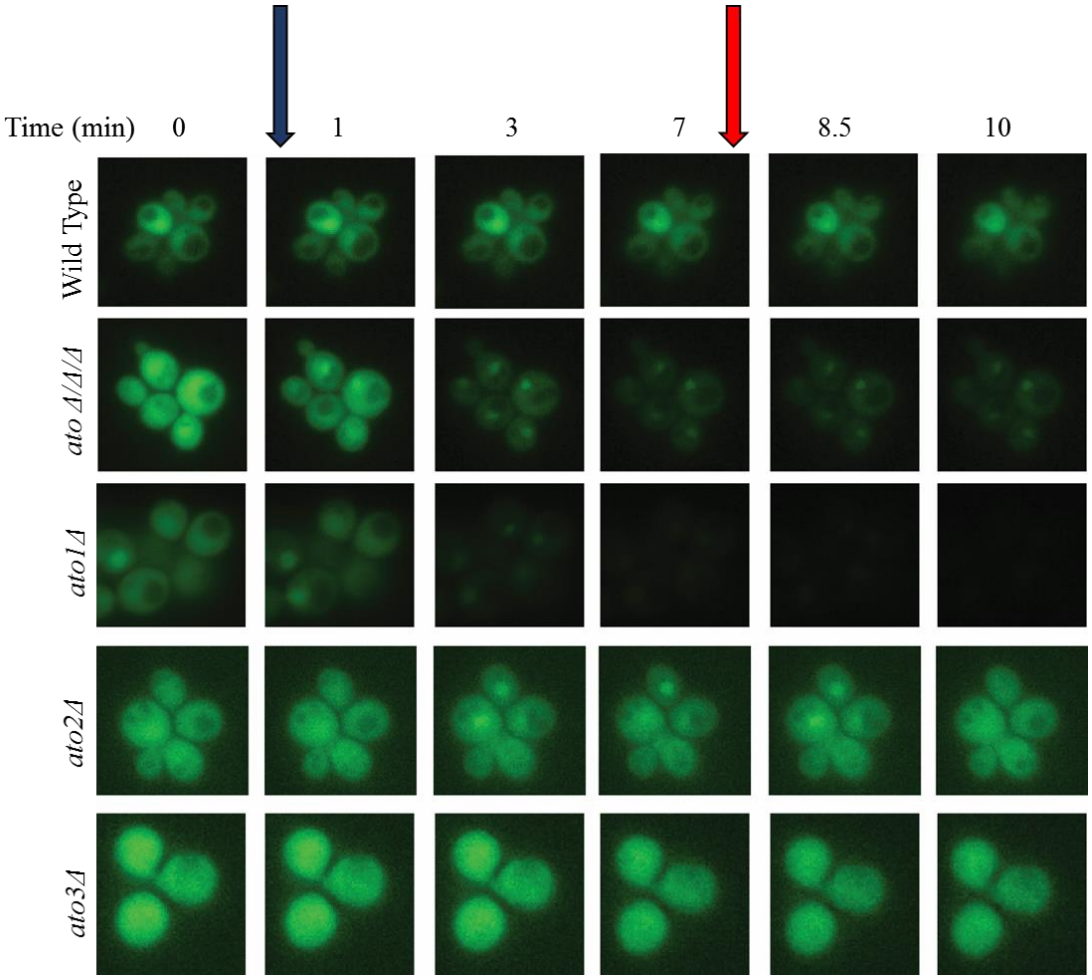

Figure 5-7. Ato1 is primarily responsible for maintenance of cytosolic pH during acetate stress. Wild-type (Σ1278b), *ato* Δ/Δ/Δ (LY342), *ato1Δ* (LY338), *ato2Δ* (LY351), and *ato3Δ* (LY355) strains expressing pHluorin were placed in a Cellasic microfluidics chamber and SD-ura+glycerol (pH 4) medium was flowed over the cells. Media was changed to SD-ura+glycerol + 0.25% acetate (pH4) at the start of the experiment (Blue arrow) and returned to SD-ura (pH 4) medium after 7 minutes (red arrow). GFP fluorescence was monitored using at 15 second intervals throughout the experiment. Representative images are presented of live cell imaging performed at least three times.

Ato proteins are necessary for utilization of amino acids in *S. cerevisiae*

Ato proteins derived their name from studies that examined extruded ammonia as a potential signalling molecule between colonies growing on plates containing glycerol and yeast extract (GM) (101, 102); however these studies have been challenging to reproduce (94). We sought to characterize amino acid utilization in *S. cerevisiae* because the small number of Ato genes reduced potential functional redundancy. Experiments designed to reproduce the reported ammonia release from *S. cerevisiae* BY4742 grown on solid GM medium were unsuccessful; however, I was able to detect neutralization of liquid GM cultures by EM93 (Figure 5-8A) Cells grew well and pH rose slowly over a period of 3 days (Figure 5-8B) compared to 6-12 hours in *C. albicans* as discussed in chapter 3. Alkalinization and growth was also observed in defined YNB+2% casamino acid medium (Figure 5-8A-B) and this medium was chosen for further experiments.

The parent strain for the Ato mutants (Σ1278b) was tested for the ability to utilize amino acids in liquid culture (Figure 5-9A-B), with similar results as EM93. Therefore, *ato* mutant strains lacking *ATO* genes were tested in liquid culture. However, deletion of any single *ATO* gene or the over-expression of the dominant negative $ATO1^{G72D}$ or $ATO2^{G72D}$ alleles abolished growth on casamino acid medium (Figure 5-9C) as did all combinations of deletions (data not shown). To confirm that this growth defect was due to an inability to efficiently utilize amino acids 2% glycerol was added to casamino acid medium to support growth. However, while the strains grew (Figure 5-10A) alkalinization remained significantly impaired with the disruption of Ato function either through deletion or overexpression of a dominant negative allele (Figure 5-10B).

Therefore, we conclude all three *S. cerevisiae* Ato proteins must be functional for efficient alkalinization in the presence of amino acids.

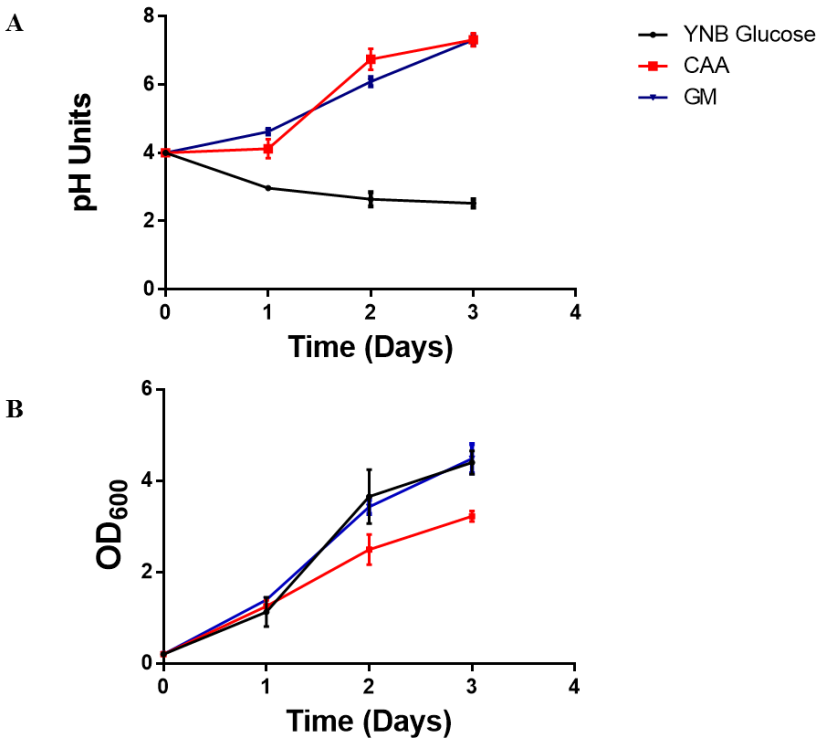

Figure 5-8 *S. cerevisiae* alkalinizes medium containing amino acids. Wild-type (EM93) cells were incubated in the indicated medium at pH 4.0 under aerated conditions at 30°C. (A) pH of the cultures from the same experiments whose results are shown in panel B. (B) Growth of the cells was measured by OD600 readings at the indicated time points. Results are reported as mean values +/- SD of triplicate assays.

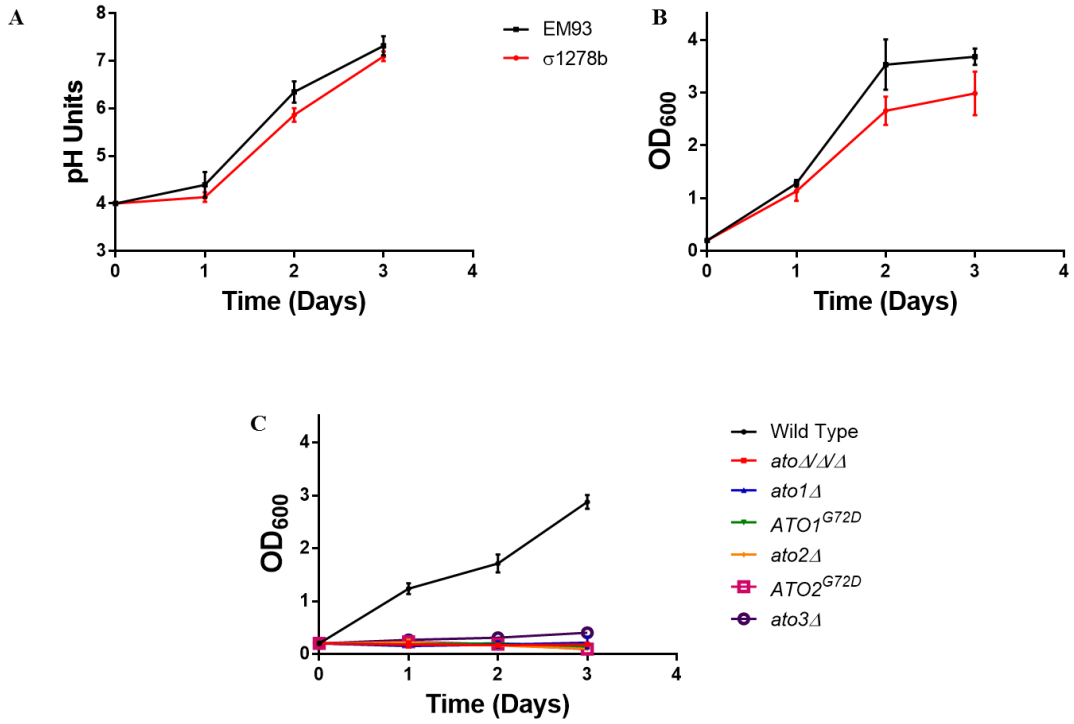

Figure 5-9 Loss of *S. cerevisiae* Ato proteins prevents growth on amino acids as a sole carbon source. Wild-type (EM93) or (Σ1278b) cells were incubated in YNB+2% casamino acids (pH 4.0) under aerated conditions at 30°C. pH (A) and growth (B) of cultures were monitored daily. (C) Indicated *ato* mutant strains along with the isogenic wild-type (Σ1278b) were incubated in YNB+2% casamino acids (pH 4.0) under aerated conditions at 30°C and growth was monitored by OD_{600} measurements. Results are reported as mean values +/- SD of triplicate assays.

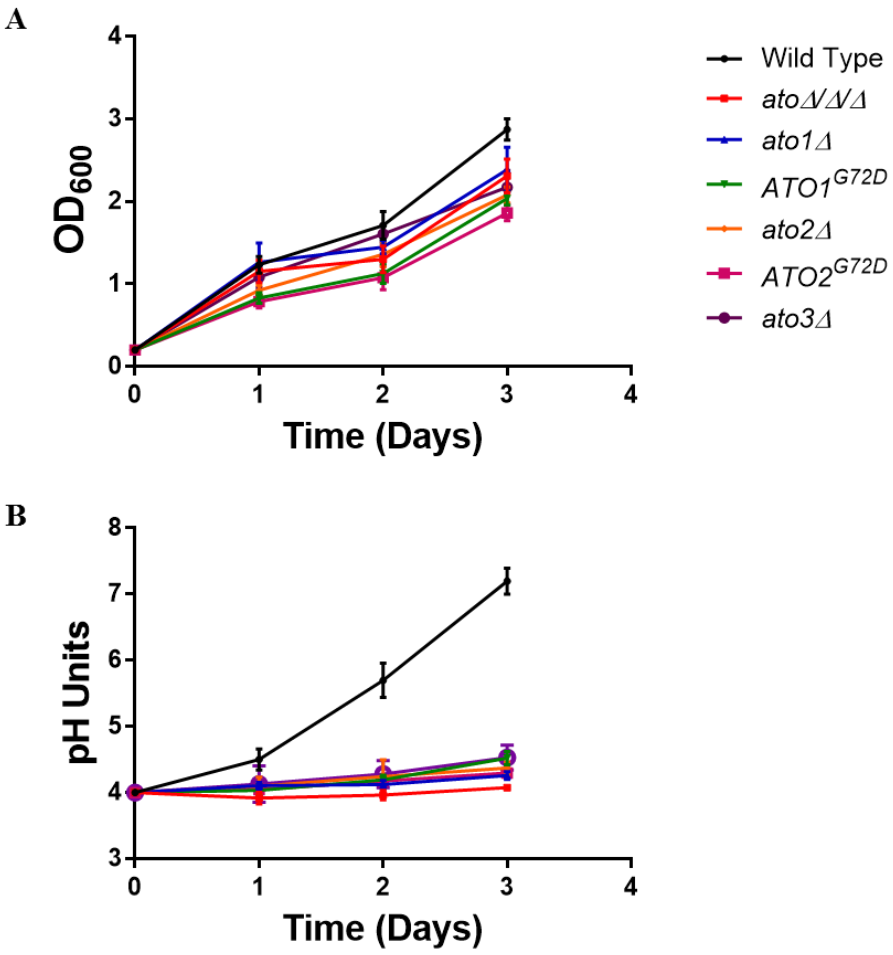

Figure 5-10 *S. cerevisiae* Ato mutants are impaired in alkalinization. Wild-type (Σ1278b) (LY40), cells were incubated in the indicated medium at pH 4.0 under aerated conditions at 30°C. (A) pH of the cultures from the same experiments whose results are shown in panel B. (B) Growth of the cells was measured by OD600 readings at the indicated time points. Results are reported as mean values +/- SD of triplicate assays.

Discussion

Ato homologs were first identified in *Y. lipolytica* where mutation of Gpr1 in the conserved FGGTLN motif conferred a dominant negative phenotype that abolished acetate utilization through an undefined mechanism (112). Previous work has implicated that Ato proteins are important for the active uptake of acetate in *S. cerevisiae* (Ato1) and *A. nidulans* (AcpA) (107, 109). However, the observed phenotypes from our previous Ato studies and those of others suggested a potential role for Ato homologs as acetate exporters that would contribute to the maintenance of cytosolic pH (114, 149). The experiments reported here aimed to elucidate the function of *S. cerevisiae* Ato proteins in the maintenance of cytosolic pH homeostasis and the utilization of amino acids as a non-fermentable carbon source.

Ato protein-protein interactions

One possible explanation for the disparate phenotypes reported for Ato mutants is that Ato proteins formed hetero-multimeric complexes with other membrane proteins. We tested this hypothesis utilizing a protein complementation assay with a wide range of candidate interactions partners and found no evidence for Ato protein interaction with heterologous proteins of the candidates chosen (Table 3-2). Ato1 forms both a homo-dimer with itself and a heterodimer with Ato2; however, it does not associate with Ato3. Ato2 and Ato3 both self-associate to form homo-dimers, similar to Ato1. The expression of a dominant negative allele of either $ATO1^{G72D}$ or $ATO2^{G72D}$ did not abolish interactions. These results are consistent with reported FLIM/FRET studies of Ato proteins in *S. cerevisiae* (103, 104). While providing more supporting evidence than the

microscopy studies this assay does not provide information on the potential higher order structures that may be formed between Ato proteins in the membrane.

Ato1 is primarily responsible for pH homeostasis during acetate stress

Previous work from our laboratory suggested that Ato proteins do not solely function as inward acetate transporters as strains over-expressing the *ATO1*^{G72D} allele fail to grow at low pH even in the presence of glucose. This evidence indicated a potential role for Ato proteins in cellular detoxification during weak acid stress (149). I hypothesized that Ato proteins function as acetate exporters and mutants would be unable to export a charged acetate anion efficiently. To further test this novel proposed function of Ato proteins as acetate exporters, liquid acetate toxicity assays on strains lacking all three *ATO* genes compared to wild-type controls were performed, revealing that *ato* Δ/Δ/Δ were sensitive to acetate stress (Figure 5-2) in a time and concentration dependent manner (Figure 5-3 A-B).

To determine if the toxicity was the result of uncontrolled acidification of the cytosol, a pH sensitive derivative of GFP was utilized to approximate cytosolic pH during live cell microscopy utilizing a microfluidics system. In agreement with the toxicity assays, cells lacking all *ATO* genes were unable to prevent the acidification of the cytosol (Figure 5-4), which supported my hypothesis that Ato proteins functioned in cytosolic homeostasis during weak acid stress, ascribing a new role to this family of proteins. In order to further elucidate the contribution of each Ato protein to cytosolic pH maintenance, strains lacking only one of the three Ato proteins or over expressing the dominant negative *ATO1*^{G72D} were challenged with 0.25% acetate stress. Strains lacking a functional Ato1 displayed severe growth impairment (Figure 5-6 C) that was not

observed in strains lacking *ATO2* or *ATO3*. These results were confirmed by live cell microscopy of strains expressing pHluorin (Figure 5-7), where acidification of the cytosol was observed in the strains lacking all three *ATO* genes and only *ATO1*, but not *ATO2* or *ATO3*. Taken together this provides strong evidence that *S. cerevisiae* Ato1 is primarily responsible for maintenance of cytosolic pH during acetate stress.

All three *S. cerevisiae* Ato proteins are required for casamino acid utilization

Despite more evidence linking homologs to acetate transport in several fungal species as discussed above, they were named for reported involvement in ammonia transport in *S. cerevisiae* (102). Additionally, it has been reported that *ATO3* is under the transcriptional control of the amino acid sensing system (176), further substantiating a role for Ato proteins in amino acid utilization. However, previous studies have only examined the deletion of a single Ato protein and never all three simultaneously. These early experiments of colonies growing on solid medium have proved challenging to reproduce. Therefore, we sought to examine pH change in liquid culture rather than solid medium, and to disrupt all three *ATO* genes simultaneously to see if we could exacerbate the modest phenotypes previously reported (101, 102).

While both EM93 and Σ1278b cells were able to raise the pH of liquid cultures utilizing casamino acids as a carbon source (Figure 5-8, 5-9 A-B), all strains with mutations in *ATO* genes failed to grow (Figure 5-9C). Even when growth was supported by glycerol, *ato* mutant strains were unable to alkalinize the media within 3 days (Figure 5-10). These data together suggest that all three Ato proteins perform non-redundant functions in amino acid utilization, although the precise mechanism of function remains unknown. Data presented in this chapter provide new insight into the relative importance

of individual Ato proteins in cytosolic pH maintenance and amino acid utilization of *S. cerevisiae* which will be further elaborated on in Chapter 6.

Chapter Six:

Discussion and Perspectives

Candida albicans is the most important health-related human-associated fungus. As a commensal microorganism it can be isolated from the gastrointestinal tract, oral cavity, skin, and genitourinary tract of healthy individuals (50). This opportunistic pathogen may cause superficial infections as well as fatal systemic infections such as invasive candidiasis (116). Systemic candidiasis is the 4th most important hospital associated blood stream infection in the United States with a high mortality rate of ~46-75% (10, 116, 117). This mortality rate is attributed in part to insufficient diagnostic tools, a lack of antifungal drugs, and the emergence of antifungal resistant strains. However, it has been established that the innate immune system function is a primary determinant of *C. albicans* disease progression, and individuals that are immunocompromised have substantially greater risk of severe infections (10). Therefore, a better understanding of interactions between C. *albicans* and the innate immune system is necessary, as innate immunity is the initial defense that the pathogen encounters in the mammalian host.

C. albicans is very adept at responding to environmental cues and employs rapid and sophisticated adaptation mechanisms facilitating both persistence and pathogenicity. Previous studies have focused upon elucidation of the role of "true" virulence factors in C. *albicans* such as hyphal morphogenesis, adherence mechanisms, biofilm formation, as well as secreted enzymes for their impact on disease progression. While these studies were critically important to understanding *C. albicans* ability to cause disease, there has been a fundamental gap in the knowledge of the critical metabolic adaptations that *C. albicans* employs. Acquisition of nutrients and the generation of cellular energy through metabolic flexibility has been demonstrated as the driving force for both commensalism

and pathogenicity; strains lacking metabolic pathways needed to assimilate amino acids, fatty acids, and other alternative carbon sources have reduced virulence (87, 90, 91, 119-122). It has also been established that nutrient limitation within the host is a defense mechanism against *C. albicans* growth, as mutants unable to produce nucleotides or import iron are avirulent (60, 177, 178). In contrast, amino acids appear to be a readily available nutrient, since many (but not all) tested mutants with auxotrophies remain fully virulent (93, 135, 179).

Transcriptional profiling and proteomic analysis have been utilized to elucidate how *C. albicans* is able to adapt to encounters with immune cells (31, 86, 87); revealing that *C. albicans* undergoes substantial transcriptional reprogramming which is specific to the type of phagocytic cell encountered. Following phagocytosis by macrophages, genes necessary for utilization of amino acids and other non-fermentable carbon sources as nutrients are strongly upregulated while glycolysis is repressed (87). Amino acids seem of particular importance as numerous oligo-peptide transporters, amino acid permeases, and degradative enzymes are induced suggesting that amino acids are an abundant nutrient (87). While amino acid utilization has been historically studied in terms of nitrogen assimilation, the work presented here focuses upon amino acids as an important carbon source for *C. albicans* in the context of interactions with phagocytes. Compared to other fungal species *C. albicans* utilizes amino acids avidly and is able to grow at nearly the same rate as during sugar utilization *in vitro*, which suggests that *C. albicans* has adapted its metabolic pathways to support efficient growth on these physiologically relevant carbon sources.

***ATO* genes promote alkalinization during amino acid catabolism**

My work has focused upon the *ATO* gene family, which has undergone dramatic expansion in *C. albicans* and a few other related pathogenic fungi relative to the model yeast *S. cerevisiae.* The studies presented here represent the first characterization of these putative ammonia/acetate transporters in *C. albicans* and identifies them as important for alternative carbon assimilation in both *C. albicans* and *S. cerevisiae*; as well as cytosolic pH homeostasis when challenged with weak acid stress. These findings represent a significant contribution to understanding how alternative carbon metabolism in fungi is linked to mechanisms of both stress response and virulence.

Clinically relevant CUG clade species, *C. albicans, C. tropicalis*, and *C. parapsilosis*, have expanded *ATO* gene families. Previous work demonstrated a positive correlation between the number of *ATO* homologs and the robustness of alkalinization (94, 180). The dramatic expansion of the *ATO* gene family strongly suggests that differential functions and/or regulation exist between them in *C. albicans*, given the potential for redundancy. My work here has demonstrated *ATO* genes are important during amino acid utilization and established that they are transcriptionally dependent upon *STP2,* the master regulator of alkalinization in *C. albicans (129).*

Through mutational analysis I demonstrated that *C. albicans ATO1* and *ATO5* both promote alkalinization during amino acid utilization; and that combinatorial functional disruption in these genes results in additive effects (94, 129). Further, overexpression analysis showed that many *ATO* genes promote environmental alkalinization in the conditions tested (129); strongly supporting the hypothesis that many *ATO* genes contribute to alkalinization and that functional redundancy contributed to the

modest phenotypes observed in single mutant strains. Intriguingly, not all *ATO* genes tested had a positive impact on alkalinization, as overexpression of *ATO3* resulted in abrogation of alkalinization and ammonia release (129), which supports the hypothesis that conservation of so many genes in a family would indicate differential functions that are not entirely overlapping.

Ammonia is a broadly conserved microbial tool

We have demonstrated ammonia release is positively correlated with neutralization of ambient pH during amino acid catabolism in *C. albicans* (94). Manipulation of phagosomal pH by microbes has been observed in both fungal and bacterial pathogens and is not unique to *C. albicans.* Neutralization of local microenvironments is utilized by the bacterial species *Helicobacter pylori.* As the causative agent of peptic ulcers, *H. pylori* utilizes extracellular ammonia production to survive the acidic environment of the stomach through the action of urease to produce ammonia (181). Additionally, *H. pylori* has been shown to utilize ammonia production to neutralize the phagolysosome of J774A.1 and murine peritoneal macrophages (182). In fungi, amino acid catabolism has been studied in conjunction with nitrogen starvation and regulation in *S. cerevisiae, Aspergillus fumigatus, Metarhizium ansipoplae, Yarrowia lipolytica,* and *Coccidioides immitis* (101, 183, 184). Taken together, this suggests that while ammonia release is broadly utilized among microbial species *C. albicans* adaptation to utilize metabolic byproducts directly to alter the extracellular environment is novel.

Amino acid utilization promotes *C. albicans* ammonia release and hyphal formation

Previous studies had established ammonia to be the effector molecule of environmental alkalinization during amino acid catabolism (94). In good correlation with the alkalinization experiments just discussed, I have demonstrated that Ato proteins facilitate the extrusion of ammonia from the cell. Mutant strains that are impaired in the ability to neutralize the environment also release less ammonia, while conversely, over-expression strains that increase alkalinization also show higher levels of ammonia release (129). These results provide strong evidence that multiple *C. albicans* Ato proteins can facilitate ammonia release, although further studies will be necessary to determine the molecular mechanism through which this process occurs.

ATO genes derived their name from observed ammonia release when the cells were grown on solid media containing glycerol and yeast extract (which contains amino acids of undefined composition) (101, 102). However, these studies did not speculate on how the released ammonia is generated. Reported experiments were performed using a common lab strain, BY4742, which harbors multiple auxotrophies (*his3Δ1, leu2Δ0, lys2Δ0, and ura3Δ0*) which could be problematic given the undefined nature of the assay medium. Though many attempts were made to recapitulate these results, no alkalinization or ammonia release by *S. cerevisiae* was observed on solid media of the same formulation by BY4742 in my hands.

However, I was able to observe neutralization of defined liquid media that contained amino acids as a sole carbon source by several *S. cerevisiae* strains that were prototrophic but not strains harboring any auxotrophies, and disruption of any of the *ATO*

genes abolished neutralization of the media (Chapter 5 and data not shown). From these results I conclude all three *S. cerevisiae ATO* genes are required for utilization of amino acids. Further, these results suggest that auxotrophies present in the BY4742 strain background impair *in vitro* alkalinization. This fits well with our model of amino acid utilization to generate ammonia relies upon the degradation of amino acids to generate energy. Strains harboring multiple auxotrophies confound the *in vitro* assay by competing the cellular needs for protein synthesis with the degradation of the same molecules for energy.

A close relative of *S. cerevisiae,* the clinically relevant *Candida glabrata,* also has 3 *ATO* homologs. Despite the smaller number of *ATO* genes compared to other pathogenic fungi (*C. albicans, C. parapsilosis,* and *C. tropicalis*) it has been reported to alkalinize the environment during amino acid utilization *in vitro* within 24 hours (as compared to 3-14 days in *S. cerevisiae*) as well as in the macrophage phagolysosome (185). Moreover, *Candida glabrata* has emerged as the second most prevalent cause of systemic candidiasis after *C. albicans (10)* which makes it intriguing to speculate that although there are only 3 *ATO* genes that they may have increased alkalinization efficiency over the same number of genes in *S. cerevisiae.* It appears that *C. glabrata* also has evolved distinct mechanisms for dealing with macrophage phagocytosis, as it has been reported that it persists and replicates within the phagosome.

The polymorphic nature of *C. albicans* is critical to virulence (62, 155). Neutral pH is a well-established signal to induce germination and our lab has shown that amino acid catabolism *in vitro* is sufficient to promote hyphal growth (94). Therefore, I hypothesized the *ATO* mutant strains would be impaired or delayed in the morphogenic

switch because of the delay in environmental alkalinization. Experiments that were included in Chapter 3 supported this hypothesis as strains growing in casamino acid medium germinated only as the pH rose to near neutral. However, experiments presented in Chapter 4 suggested that it was not only the neutrality of the environment alone but also the presence and/or degradation of amino acids that trigged morphogenesis. *C. albicans* cells grown in medium containing α-ketoglutarate rapidly raised the culture pH but no germination was observed unless amino acids were present in the medium. However, it remains to be elucidated if the combination of the media acts as an inducer of hyphal morphogenesis directly, or perhaps overcomes some sort of repression of filamentation that is exerted by the presence of carboxylic acids. In fact, it has been suggested that the presence of extracellular amino acids (specifically proline or methionine) in the culture medium may stimulate hyphal growth although glutamate has not been individually tested for induction of filamentation (50).

Hyphal formation in *C. albicans* has long been associated with tissue invasion and virulence. *C. albicans* is able to readily form filaments within macrophages which facilitates escape from the phagocyte. We have shown that *C. albicans* can actively neutralize the phagosome and thus auto-induce its own morphogenic switch (94), and strains lacking *STP2* do not germinate as well wild-type cells following phagocytosis (97). I hypothesized that the defects in alkalinization and ammonia release of *ato* mutant strains would also result in a reduction in macrophage cytotoxicity as a consequence of inhibition of hyphal formation. Experiments in chapter 3 substantiated this prediction and provided evidence that links *C. albicans* metabolism of amino acids directly to fitness within macrophages (129).

However, despite compromised interactions with macrophages no significant attenuation in virulence was observed in a mouse model of disseminated infection. It is logical that pathogens would expand gene families necessary to facilitate specific interactions with the host, however this historically has been challenging to demonstrate experimentally due to technical limitations in knocking out multiple genes. Examples of this struggle are illustrated by *C. albicans* secreted aspartyl proteases (SAPs) that each have widely variant expression profiles and pH optima, but conclusive evidence of individual genes' contribution to pathogenesis has been elusive (77, 186, 187). Als3, one of a family of eight adhesins, has several important *in vitro* phenotypes such as iron uptake, adhesion, and cadherin-binding, but is dispensable for virulence in whole animal models(68, 188, 189). Thus, we conclude that the most likely explanation is that functional redundancy of the Ato proteins explains the lack of *in vivo* virulence defects (129). This explanation is also supported by the fact that *stp2Δ* cells have only a modest attenuation in virulence (97) and yet should have significantly reduced *ATO* gene expression (129). The ability to more easily make multiple *ATO* mutants in an efficient manner has just been revolutionized in *C. albicans* with the very recent adaptation of the CRISPR/Cas9 gene editing system (190), which will significantly reduce the time needed to make combinatorial deletions and reduce functional redundancy.

I have demonstrated that over-expression of several *ATO* genes enhances the ability of the cell to alkalinize the extracellular space and excrete ammonia. However, *ATO3* over-expression strikingly resulted in inhibition of both alkalinization and ammonia release, suggesting a potential role of a negative regulator which warrants future studies. An alternative explanation for the loss of alkalinization is that a precise

stoichiometry of different Ato proteins must be maintained in order to function properly in a hetero-multimeric complex, and that too much *ATO3* disrupted the balance. The protein complementation assay presented in chapter 5 demonstrates that *S. cerevisiae* Ato proteins form complexes. This evidence is in good agreement with other from studies done in *S. cerevisiae* and also in *Y. lipolytica ATO* homolog *GPR1* (103, 104, 114).

While the work presented here begins to characterize the *C. albicans ATO* proteins there are many more unanswered questions that remain. We have identified *STP2* as important for induction during amino acid utilization, however RNA-seq data suggests that some *ATO* genes are still expressed at low levels even in the absence of *STP2,* and what transcription factor(s) are responsible for that expression remain unknown (H. Danhof, S. Vylkova, and M. Lorenz unpublished observations). It is intriguing to speculate that because of the characterization as acetate transporters in other microbial species, *ATO* genes will also be subject to regulation based upon the presence of organic acids as well as amino acids, making the transcription factor *RGT1, (R*epressor of Glucose Transport, named for its function to repress hexose transporters) and attractive additional potential positive regulator of *ATO* genes.

Equally importantly, information is lacking regarding the transcriptional repression or post-transcriptional regulation of these genes. The most obvious target as a possible transcriptional repressor would be *RIM101,* which is the master regulator for the alkaline tolerance response of *C. albicans.* Alkalinization from acidic to neutral in *C. albicans* is independent of *RIM101*(94), however while environmental alkalinization is induced in response to availability of amino acids through *STP2,* it could be repressed through means of pH adaptation in addition to carbon source availability.

The work presented here represents the entirety of current knowledge about *C. albicans ATO* function, which I have established as important for amino acid utilization. However, there is much more support for a role of Ato proteins as acetate transporters. The dominant negative *ATO1*^{G53D} mutant was originally identified in *Y. lipolytica*, conferring sensitivity to acetic acid, as do similar mutations in *S. cerevisiae* as discussed in chapter 5 (112, 114). Ato proteins in *Aspergillus nidulans* (AcpA) and Ato1 (Ady2) in *S. cerevisiae* facilitate active transport of acetate (107, 191) and the *E. coli* homolog SatP/YaaH is an acetate/succinate transporter (192). Trying to reconcile the disparate phenotypes reported for Ato proteins is technically challenging in *C. albicans* due to the more complex genetic manipulations and the potential for redundancy between the Ato proteins.

Ato protein-protein interactions

These studies were initially prompted by the observed extrusion of ammonia by *C. albicans* cells during amino acid utilization as described above. However, *S. cerevisiae* with only three *ATO* genes is much more manageable in terms of mutant analysis. Therefore, I chose to instead characterize *S. cerevisiae* for acetate toxicity. One possible explanation for the disparate phenotypes reported for Ato mutants is that Ato proteins formed multimeric complexes with themselves or other membrane proteins. As described in Chapter 5, Ato1 forms both a homo-dimer with itself and a hetero-dimer with Ato2; however, no association with Ato3 was observed. Ato2 and Ato3 both self-associate to form homo-dimers, similar to Ato1. The expression of a dominant negative allele of either *ATO1*^{G72D} or *ATO2*^{G72D} did not abolish interactions. These results are consistent with reported FLIM/FRET studies of Ato proteins in *S. cerevisiae* and provide more

evidence of interactions between Ato proteins (103, 104). These findings are consistent with reported indications that the *ATO1* homolog in *Y. lipolytica*, *GPR1*, also forms oligomeric structures (113, 114). These findings suggest that through oligomerization, Ato proteins potentially form structures that more closely resemble other transport proteins such as the 12 transmembrane domains of carboxylic acid transporters or the 10-12 membrane spanning domains of ammonium permeases and match the predicted functions of the proteins. Additionally, the potential for hetero-multimeric complexes with a subset of candidate interactions partners that were involved with transport of nutrient acquisition in high throughput screens (172, 173). No interactions were detected; however, this assay was designed to target a handful of rationally chosen interaction partners and thereby there are many potential interaction partners that were not tested and cannot be ruled out, and will be discussed in the future directions section.

Previous work from our laboratory indicated that Ato proteins may function in acetate efflux, as strains over expressing the $ATO1^{G72D}$ allele exhibited acetate toxicity consistent with an inability to efflux a charged acetate anion efficiently (149). It has been reported by several studies that homologs of Ato proteins are involved in acetate uptake and utilization by fungal species, as discussed in the introduction (107, 109, 112). However, definitive biochemical validation of *S. cerevisiae* Atos solely as inward transporters has not been established. I hypothesized a novel function for the ATOs as acetate exporters crucial for maintaining cytosolic pH homeostasis during weak acid stresses. In order to test this hypothesis, I performed liquid acetate toxicity assays of strains lacking *ATO* genes and single cell fluorescence microscopy of these mutants utilizing a pH sensitive variant of GFP, pHluorin. These assays revealed that Ato1

function is crucial for acetate stress resistance and cytosolic pH maintenance although the mechanism of Ato function remains elusive and will be discussed in the future directions section.

Perspectives and future directions

At the end of all the work described here I am left with far more open questions than concrete answers. These questions largely stem from the indirect nature of most of the current assays performed, which makes inferring molecular function impractical and difficult to support. This is further compounded by the fact that these studies have been conducted in two related yeast species that occupy very different lifestyles, and thereby "go about their business" of daily life quite differently. One simple illustration of this point is how differently *C. albicans* and *S. cerevisiae* react to normal human temperature. For *S. cerevisiae* 37°C is a thermal stress, while it is a normal growth temperature of *C. albicans* that has adapted to life within a mammalian host. On the surface, both organisms survive the same temperature conditions but the detailed responses the stimulus are dissimilar upon closer inspection.

Therefore, the future directions proposed below outline experiments to be performed in either *S. cerevisiae* or in *C. albicans* with very little direct overlap. At this early point in the overall study of the Ato family I feel it is a "necessary evil" to simply acknowledge that while the basal molecular function of Ato proteins is almost certainly conserved between *S. cerevisiae* and *C. albicans* (as well as other fungi and bacteria) the divergence between these organisms has altered (likely specialized) Ato function. *C. albicans* utilizes amino acids for growth much more robustly than *S. cerevisiae* does, I would speculate at least in part due to the expansion of the *ATO* gene family, making

direct metabolic comparison impractical. However, studies in *C. albicans* are complicated by an overwhelming potential for redundancy in Ato function, making studies in *S. cerevisiae* the most logical choice to try and ascertain direct transport function. While investigating function in two species often makes assay comparison challenging, it also provides a robust pool of experiments that can be performed, that I believe will ultimately aid in elucidation of the molecular and biological functions of the *ATO* gene family.

What do Ato proteins directly transport?

S. cerevisiae strains lacking a fully functional Ato1 protein (*ato1Δ, ATO1*^{G72D}, or *ato Δ/Δ/Δ*) all exhibited acetate toxicity and a loss of cytoplasmic pH homeostasis when challenged with acetate at low pH; however, whether these phenotypes are direct or indirect remains elusive. The simplest explanation of these correlated phenotypes is that Ato1, either individually or in concert with other Ato proteins, can efflux acetate thereby detoxifying the cytosol of the accumulation of charged anion. The most direct way to test this hypothesis would be to perform efflux assays of cells preloaded with radiolabeled acetate. If Ato proteins function to efflux the anion from the cell, then mutant cells lacking functional Ato proteins would accumulate the anion while wild-type cells would be fully able to export it. Based upon the data presented here Ato1 is primarily responsible for this efflux and would be predicted to have the greatest acetate accumulation. In this model anion efflux would need to be coupled to removal of a proton from the cytosol to prevent acidification, probably through the action of the major plasma membrane proton pump, Pma1. To test if Pma1 was involved, inhibiting proton pumping

using a chemical inhibitor (such as diethylstilbestrol) would increase the acidification of the cytosol and reduce acetate efflux in both Ato mutants and wild-type cells.

Though the simplest explanation with regards to acetate transport and toxicity, the above model does not sufficiently account for the rise in extracellular pH that is promoted by Ato proteins during amino acid utilization in *S. cerevisiae* and *C. albicans.* While ammonia release in *C. albicans* is correlated with amino acid degradation and the extrusion of volatile ammonia from the cell, there is no direct evidence suggesting they are ammonia/ammonium transporters. The initial suggestion of this function was published by the Palkova group that found a minimally conserved ammonium transport signature from *C. elegans* in *S. cerevisiae* Ato1 (102). While this bioinformatics link is tenuous at best, the reported ammonia release defects of *ATO* mutants suggested the hypothesis was plausible. I performed Phyre2 in silico analysis on *S. cerevisiae* and *C. albicans* Ato1 proteins and the only transport signature similarity observed was a putative voltage-gated chloride channel. Taken together these signatures suggest the possibility of direct co-transport of a small positively charged and a small negatively charged molecule, perhaps ammonium and chloride or possibly ammonium with acetate. Direct facilitated co-transport of small molecules is highly dependent upon the concentration gradient to drive molecular movement. Therefore, one simple way to test this possibility would be to increase the extracellular ion concentrations (either ammonium or chloride) and determine if there is an impact on the transport of radiolabeled acetate either into or out of the cell in the case of *S. cerevisiae.* Since acetate transport by *C. albicans* Ato proteins has not been tested, a better assay would perhaps be to increase extracellular chloride

concentrations in defined casamino acid medium and ascertain if there is suppression or exacerbation of the alkalinization phenotypes of *ato* mutant strains.

A third possibility for Ato function that could account for the varied phenotypes is through interactions with heterologous membrane proteins. While the protein complementation assay in *S. cerevisiae* did not suggest any interactions apart from other Ato proteins, the number of candidates tested was relatively small and focused upon nutrient transporters. The dominant negative effects of the $ATO1^{G73D}$ allele suggest that complex formation or substrate binding is impaired in these strains, which indicates that the N-terminal domain may regulate coordinated transport of acetate or ammonium without directly transporting the substrate. Good potential candidates for further studies include the plasma membrane proton pump, Pma1, and the known carboxylic acid export protein, Pdr12.

The first step in testing this model is to determine if the Ato proteins are in close proximity for interactions with the candidate proteins. Since Ato1 forms patches at pH over 6, and is dispersed in the plasma membrane at pH <5, it may be beneficial to perform microscopic co-localization experiments utilizing fluorescently tagged proteins in a microfluidics chamber and changing the pH of the media. While not as definitive as FRET or FLIM analysis, co-localization studies are relatively easy to perform and could be followed by more detailed protein interaction studies. If membrane co-localization is observed, then mutant analysis could be performed for exacerbation or suppression of acetate toxicity phenotypes as described above.

Ato homologs in *S. cerevisiae* and *A. nidulans* have been characterized as inward acetate transporters; however, at low extracellular pH uncharged acetic acid can freely

diffuse into the cytosol and deprotonate in the neutral cytoplasm. This generates a weak acid stress to remove acetate that cannot immediately be metabolized by the cell. Our data support the hypothesis that *S. cerevisiae* may also function to export anions from the cell to prevent accumulation, although the mechanism remains elusive. A model of the three potential mechanisms of Ato protein function is illustrated in Figure 6-1.

How are *ATO*s regulated?

ATO gene expression is subjected to catabolite repression by the presence of glucose as was discussed in the introduction, which is typical of genes involved in alternative carbon metabolism. However, the rapidly changing nutrient environment within the human host, suggests that *C. albicans* would benefit from extremely rapid metabolic adaptation mechanisms. Possible clues to post-transcriptional regulation may be found in work that suggests *Y. lipolytica GPR1* is regulated through phosphorylation events at serine residues in a carbon source dependent manner (113). This sort of post-transcriptional regulation makes more sense from a cell energetics view than wholesale transcriptional induction and repression when the environmental conditions within the host are continually changing. Further, this hypothesis is strengthened by the recent discovery that contrary to *S. cerevisiae,* C. *albicans* retains enzymes necessary for the utilization of lactic acid and other physiologically relevant carbon sources even when glucose is available (193). This regulation promotes colonization of niches where the nutrients are

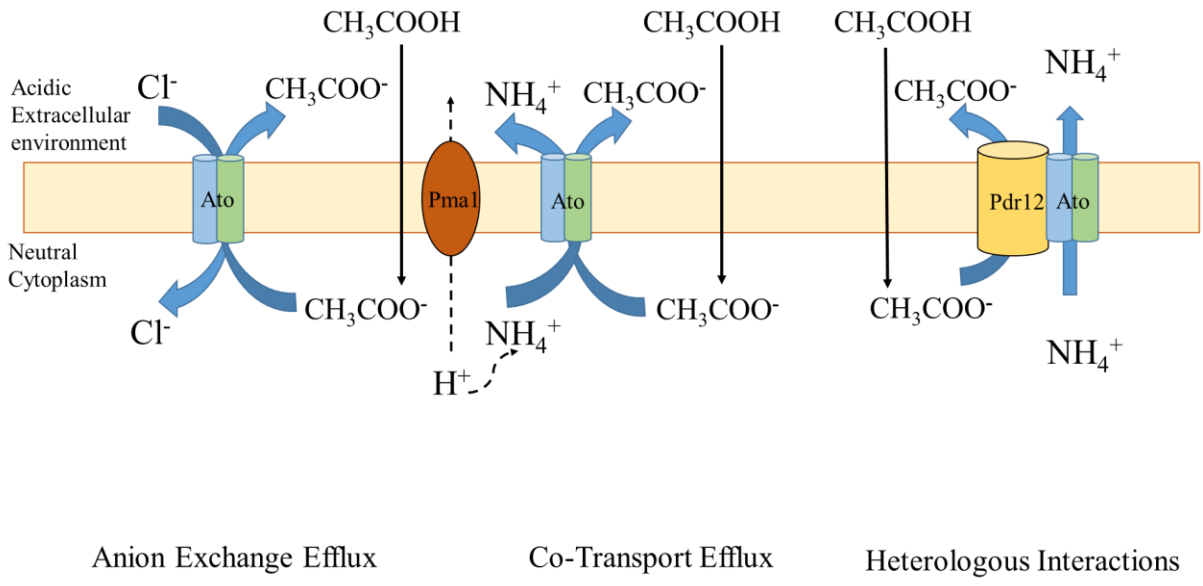

Figure 6-1. Model of potential Ato protein function in membrane transport. Presented here are three possible mechanisms that could facilitate acetate efflux: Anion exchange: where a negatively charged non-toxic chloride molecule is exchanged for the weak acid acetate. The resulting proton then is either refluxed by the plasma membrane proton pump, Pma1 or combines with an ammonia molecule and is exported from the cell by an Ato protein as ammonium. Co-transport predicts that acetate and ammonium are exported from the cell concurrently by Ato proteins. In the heterologous interaction model Ato proteins facilitate the efflux of acetate through a protein-protein interaction with the carboxylic acid efflux protein, Pdr12.

subject to transient changes, and likely facilitates rapid responses to danger signals such as macrophage engulfment. Ato proteins have several conserved serine residues in the N-terminus of the protein that may serve as potential phosphorylation sites providing potential for post-transcriptional regulation of Ato function in *C. albicans*. Interestingly, these sites are not identical in all *ATO* genes therefore mutational analysis involving both systematic truncation of the N-terminus of the protein and site directed mutagenesis of serine residues to alanine could be performed. These strains could then be tested for defects in alkalinization and ammonia release in *C. albicans.*

How to unravel the potential redundancy and elucidate specialization of Ato proteins?

The functional redundancy and potential specialization between *C. albicans* Ato proteins remains a mystery. Aside from potential differential phosphorylation events discussed above, it would be difficult to unravel the interconnectedness of this large family except for making multiple mutants. Until very recently, it was impractical to consider making a 10-mer mutant in *C. albicans* as it would necessitate 20 sequential transformations targeting 10 different genes utilizing a single recyclable drug marker. The risk-benefit analysis of this undertaking was not favorable. However, the recent adaptation of the CRISPR/Cas9 gene editing system for *C. albicans* (190) greatly improves the prospects of elucidating individual functions of Ato proteins through multiple deletions.

Overall, the big picture of this project relates back to the concept that metabolic flexibility and utilization of non-fermentable carbon sources is necessary for *C. albicans* persistence and pathogenicity. Utilization of amino acids, fatty acids, lactate, and acetate

are required for full virulence (87, 90, 91, 119, 120). Additionally, cell wall structure, hyphal morphogenesis, and stress responses are regulated by carbon source availability (146, 194, 195), highlighting the broad impact of these studies to *C. albicans* fitness within the host. Catabolism of amino acids allows *C. albicans* to modulate pH of the extracellular environment in a process which involves several Ato proteins. Alkalinization has been demonstrated to be important for interactions with macrophages, which are key components of the innate immune system. It is my hope that these studies provide a basis for future studies to elucidate how Ato proteins function in amino acid utilization and cellular pH homeostasis, which is likely broadly conserved among fungi.

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VITA:

Heather Ann Danhof (maiden: Richardson) was born on January 2, 1976, to her parents William Bradley Richardson and Ruth Ellen Richardson (maiden: Cunningham), in Muskegon, Michigan. She graduated with honors in the top 5% of her class at Muskegon High School in June, 1994 having accumulated 27 college credits concurrently while obtaining her high school diploma. She gained life experience through raising her family and work for the next twelve years, prior to returning to college in the Fall of 2006, to study Cell and Molecular Biology and Grand Valley State University. During her undergraduate studies Heather was honored to be a Ronald E. Mc Nair Scholar which opened her eyes to the beauty of scientific research and the importance of high quality college educators. In May of 2010, Heather graduated with her Bachelors of Science degree. That fall, passionate about both science and education, she entered graduate school with the full support of her husband, three daughters, mother-in-law, cat and dog; whom had all traversed the country so that she could and join the distinguished department of Microbiology and Molecular Genetics at the University of Texas Health Science Center at Houston in pursuit of her Ph.D. In the spring of 2011, she joined the lab of Dr. Michael Lorenz who diligently mentored her in both good science and dry humor. She will defend her dissertation in April, 2016 and officially graduate in May, 2016.