

UC Irvine

UC Irvine Electronic Theses and Dissertations

Title

Exploring Candida biology through integration of proteomic and genetic approaches

Permalink

<https://escholarship.org/uc/item/4gm6x5wx>

Author

Alkafeef, Selma

Publication Date

2018

Copyright Information

This work is made available under the terms of a Creative Commons Attribution License, available at <https://creativecommons.org/licenses/by/4.0/>

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA,
IRVINE

Exploring *Candida* biology through integration of proteomic and genetic approaches

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Biomedical Sciences

by

Selma S. Alkafeef

Dissertation Committee:
Professor Haoping Liu, Chair
Professor Lan Huang
Professor Peter Kaiser
Associate Professor Feng Qiao
Professor Ken W. Y. Cho

2018

DEDICATION

To my parents,
for their unending love and support.

Think left and think right and think low and think high.

Oh, the thinks you can think up if only you try!

- Dr. Seuss

TABLE OF CONTENTS

	Page
LIST OF FIGURES	iv
LIST OF TABLES	vi
ACKNOWLEDGMENTS	vii
CURRICULUM VITAE	ix
ABSTRACT OF THE DISSERTATION	xii
CHAPTER 1: Introduction	1
CHAPTER 2: Methods	14
CHAPTER 3: Wor1 establishes opaque cell fate through inhibition of the general co-repressor Tup1 in <i>Candida albicans</i>	32
CHAPTER 4: The glutaredoxin Grx3 is a global iron sensor and regulator of multiple iron-sulfur cluster-dependent pathways in <i>Candida albicans</i>	69
CHAPTER 5: Summary and Conclusions	105
REFERENCES	109

LIST OF FIGURES

	Page
Figure 3.1 Identification of Wor1 interacting proteins by tandem affinity HBH purification coupled with mass spectrometry	36
Figure 3.2 Tup1 binds along the <i>WOR1</i> promoter differentially in white and opaque phases	42
Figure 3.3 Tup1 levels are similar in white and opaque cells	43
Figure 3.4 Verification of <i>pMET3-TUP1</i> construct and Tup1 protein stability	45
Figure 3.5 Tup1 is a repressor of the opaque state	46
Figure 3.6 Tup1 depletion bypasses the requirement for Wor1 in the expression of <i>WOR1</i> and key opaque regulators	51
Figure 3.7 Tup1 depletion stabilizes the opaque state even at 37°C	53
Figure 3.8 Non-glycolytic carbon sources alter Tup1 occupancy at the <i>WOR1</i> promoter and stabilize the opaque phase at 37°C in <i>MTLa/a</i> and <i>a/a</i> cells	57
Figure 3.9 Protein levels of Wor1 and Tup1 remain constant when cultured in non-glycolytic carbon sources	60
Figure 3.10 A model for Tup1-mediated regulation of cell fate at the <i>WOR1</i> promoter	63
Figure 4.1 Grx3 is necessary for iron responsive regulation of the iron homeostasis circuit	71
Figure 4.2 Grx3 interacts with Sfu1 to regulate its activity at the <i>SEF1</i> promoter	78
Figure 4.3 Grx3 regulates Sef1 localization and Hap43 activity	80
Figure 4.4 The Grx3-HBH tag is functional	85

Figure 4.5	Grx3 interacting proteins display diverse functions and contribute to a wide array of biological processes	91
Figure 4.6	Grx3 plays roles in nitrosative stress response, DNA damage pathway, and translational efficiency	95
Figure 4.7	Grx3 is a global iron sensor and Fe-S carrier critical for the function of multiple Fe-S protein-dependent pathways	100

LIST OF TABLES

	Page
Table 2.1 Strains used in Chapter 3	26
Table 2.2 Strains used in Chapter 4	27
Table 2.3 Primers used in Chapter 3	29
Table 2.4 Primers used in Chapter 4	31
Table 3.1 Wor1 interacting proteins identified by mass spectrometry	37
Table 4.1 Grx3 interacting proteins identified by mass spectrometry	87

ACKNOWLEDGMENTS

Firstly, I would like to acknowledge the people responsible for all my successes in life, my parents, Saad and Cornelia Alkafeef. Their constant support and encouragement cannot possibly be quantified. I like to think that, as a close family of three, we do everything as a team. The completion of my PhD was most definitely a team effort. Thank you for all the support and love you provide me; this would not have been possible without you.

I would also like to extend my greatest gratitude to my mentor and advisor, Dr. Haoping Liu, who took a chance on a last minute rotation student in Spring 2011. Haoping has proven to be a wellspring of knowledge and advice, and through her mentorship and guidance I have developed into a better thinker and scientist. Her emphasis on addressing critical questions with elegant genetic approaches versus “busy work” is something that I will take with me through the rest of my academic career. Haoping has always kept my personal and professional goals foremost, and for that I am very grateful.

I would also like to thank members of the Liu lab, former and current, for their thoughtful discussions and support during my time in the lab. This includes Dr. Zhiyun Guan, Dr. Scott Stevenson, Dr. Yang Lu, Dr. Chang Su, Dr. Fuqiang Wang, Dr. Ohimai Unoje, Mengli Yang, Dr. Yi Sun, Dr. Jingwen Tan, Shelley Lane, Dr. Tingting Zhou, and Dr. Lingmei Sun. Whether by going through a protocol with me, sitting through long lab meetings helping me make sense of my projects, or brainstorming the best way to tackle our departmental dessert challenge, Liu lab members (honorary Liu lab member BananaDog included) have been there for me through it all. I would also like to acknowledge my collaborator from the Huang lab, Dr. Clinton Yu, who is always willing to run more samples or provide additional analysis at a moment’s notice.

I would like to thank my committee members, Drs. Peter Kaiser, Feng Qiao, Ken Cho, and my collaborator and committee member Dr. Lan Huang. Your insights and suggestions on my projects have proven exceedingly helpful and astute, and my projects are all the better for your input.

I would like to extend my deepest gratitude to the Kuwait Ministry of Higher Education, Kuwait University, and to the Kuwait University Faculty of Medicine Biochemistry Department. Few in my position can say they have received the benefits and privileges I have thanks to the generosity of the Kuwait MOHE and Kuwait University.

No acknowledgement section would be complete without mentioning my wonderfully supportive and caring network of friends, who have been through the trenches with me and truly understand the highs and lows associated with graduate life.

I wish to express special appreciation for my friends, department mates, and confidants who have supported me throughout the years. The role your support and encouragement has played in my personal and professional lives cannot be overstated. As the brilliant metaphysical poet John Donne wrote, no man is an island. This is true in science and in life.

Finally, I would like to thank the Public Library of Science (PLOS) for allowing me to reprint published materials. I would also like to thank the agencies that provided the financial support that made the work presented in this dissertation possible. This work was funded in part by the National Institutes of Health Grants R01AI099190 and R01GM117111 to Dr. Haoping Liu and a graduate scholarship from Kuwait University Faculty of Medicine to Selma S. Alkafeef.

CURRICULUM VITAE

Selma S. Alkafeef

EDUCATION

Doctor of Philosophy in Biomedical Sciences University of California, Irvine	2018
Master of Science in Biomedical Sciences University of California, Irvine	2011
Bachelor of Science in Biology Concentration in Cellular, Molecular, and Developmental Biology University of Washington	2009

RESEARCH EXPERIENCE

Graduate Researcher Advisor: Dr. Haoping Liu Department of Biological Chemistry University of California, Irvine	2011 - present
Lab Trainee Advisor: Dr. Moussa Alkhalaf Biochemistry Department Kuwait University School of Medicine	Apr. 2010 – Jul. 2010
Undergraduate Researcher Advisor: Dr. Jennifer Nemhauser Department of Biology University of Washington	Apr. 2009 – Sept. 2009
Undergraduate Researcher Advisor: Dr. Wesley C. Van Voorhis Department of Biochemistry University of Washington	Sept. 2007 – Jan 2009

TEACHING EXPERIENCE

Teaching Assistant
University of California, Irvine
BIO SCI 97: Genetics

Summer 2016

PUBLICATIONS

Hu Y, Pioli PD, Siegel E, Zhang Q, Nelson J, Chaturvedi A, Mathews MS, Ro DI, **Alkafeef S**, Hsu N, Hamamura M, Yu L, Hess KR, Tromberg BJ, Linskey ME, Zhou YH. (2011) EFEMP1 suppresses malignant glioma growth and exerts its action within the tumor extracellular compartment. *Mol Cancer*, 10(1), 123. Doi:10.1186/1476-4598-10-123

Alkafeef SS, Yu C, Huang L, Liu, H. (2018) Wor1 establishes opaque cell fate through inhibition of the general co-repressor Tup1 in *Candida albicans*. *PLOS Genet*, 14(1), e1007176

Alkafeef SS, Lane S, Zhou T, Yu C, Solis NV, Filler SG, Huang L, Liu H. The glutaredoxin Grx3 is a global iron sensor and regulator of multiple iron-sulfur cluster-dependent pathways in *Candida albicans*. *Manuscript in preparation*.

AWARDS & SCHOLARSHIPS

Kuwait University Scholar	2012 – 2018
ASM Student Travel Grant	2016
UCI School of Medicine Travel Award	2016
Kuwait Ministry of Higher Education Merit Scholarship	2005 – 2009

INVITED TALKS AND POSTER PRESENTATIONS

14th ASM Conference on Candida & Candidiasis Apr. 2018
Providence, Rhode Island
Poster presentation
Title: The glutaredoxin Grx3 is a global iron sensor and regulator of multiple iron-sulfur cluster dependent pathways in *C. albicans*

13th ASM Conference on Candida & Candidiasis Apr. 2016
Seattle, Washington
Oral presentation
Title: Wor1 sustains opaque stability through inhibition of the repressor Tup1

Department of Biological Chemistry Seminar Series

Mar. 2016

University of California, Irvine

Oral presentation

Title: A new mechanism of regulation of phenotypic switching in the pathogenic yeast

Candida albicans

10th Cold Spring Harbor meeting on Microbial Pathogenesis and Host Response

Sept. 2015

Cold Spring Harbor, New York

Poster presentation

Title: Characterization of Wor1 function through identification of interacting proteins

Department of Biological Chemistry Seminar Series

Nov. 2014

University of California, Irvine

Oral presentation

Title: Characterization of the *Candida albicans* white-opaque regulator Wor1 through identification of interacting proteins

12th ASM Conference on Candida and Candidiasis

Mar. 2014

New Orleans, Louisiana

Poster presentation

Title: Characterization of Wor1 function through identification of interacting proteins

LEADERSHIP

Graduate Student Representative

2014

Department of Biological Chemistry

University of California, Irvine

ABSTRACT OF THE DISSERTATION

Exploring *Candida* biology through integration of proteomic and genetic approaches

By

Selma S. Alkafeef

Doctor of Philosophy in Biomedical Sciences

University of California, Irvine, 2018

Professor Haoping Liu, Chair

Candida albicans is a common opportunistic fungal pathogens of humans. As such, it faces challenges unique to host-dwelling organisms and has evolved mechanisms that allow it to thrive in the constantly changing environment of the human host. In this dissertation we sought to address two such mechanisms of *C. albicans* biology.

C. albicans can undergo phenotypic switching between two heritable states: white and opaque. This phenotypic plasticity facilitates its colonization in distinct host niches. The master regulator *WOR1* is exclusively expressed in opaque phase cells. Positive feedback regulation by Wor1 on the *WOR1* promoter is essential for opaque formation, however the underlying mechanism of how Wor1 functions is not clear. In Chapter 3, we use tandem affinity purification coupled with mass spectrometry to identify Wor1-interacting proteins. Tup1 and its associated complex proteins are found as the major factors associated with Wor1. Tup1 occupies the same regions of the *WOR1* promoter as Wor1 preferentially in opaque cells. Loss of Tup1 is sufficient to

induce the opaque phase, even in the absence of Wor1. This is the first such report of a bypass of Wor1 in opaque formation. These genetic analyses suggest that Tup1 is a key repressor of the opaque state and Wor1 functions to alleviate Tup1 repression at the *WOR1* promoter. Opaque cells convert to white *en masse* at 37°C. However, we find this conversion occurs only in the presence of glycolytic carbon sources, and the opaque state is stabilized when cells are cultured on non-glycolytic carbon sources, even in an *MTLa*/α background. We further show that temperature and carbon source affect opaque stability by altering the levels of Wor1 and Tup1 at the *WOR1* promoter. We propose that Wor1 and Tup1 form the core regulatory circuit controlling the opaque transcriptional program. This model provides molecular insights on how *C. albicans* adapts to different host signals to undergo phenotypic switching for colonization in distinct host niches.

Iron is an essential molecule involved in a myriad of biological processes. Despite its essential role as a cofactor, excess iron can become toxic through the generation of reactive oxygen species. As a fungal pathogen of humans, *C. albicans* is subject to a wide range of iron levels encountered in the human host. Consequently, iron homeostasis is essential for survival and is tightly controlled by a regulatory circuit. Glutaredoxins are a conserved family of proteins involved in maintaining cellular redox homeostasis as well as the biogenesis of iron-sulfur clusters, cofactors linked to diverse biological processes including metabolism, DNA maintenance, transcriptional regulation, and protein translation. In Chapter 4, using a combination of functional genetics, molecular biology, and cross-linked tandem affinity purification coupled with mass spectrometry, we assess the function of the conserved monothiol glutaredoxin Grx3. We

examined the function of four identified *C. albicans* glutaredoxins in response to oxidative and iron stresses and found a *grx3* mutant to be sensitive to iron level. The *grx3* mutant was defective in the expression of the iron-regulatory circuit genes Sfu1, Sef1, and Hap43 in response to iron status. We determined that Grx3 interacts with Sfu1 and regulates its occupancy at *SEF1* promoter. Grx3 was also found to contribute to Sef1 nuclear localization and Hap43 activity. Therefore, Grx3 directly controls the activity of the iron homeostasis regulatory circuit. Identification of Grx3 interacting proteins by mass spectrometry uncovered proteins enriched for several functional categories, including those involved in various metabolic and biosynthetic pathways such as redox homeostasis, amino acid and nucleotide metabolism, protein translation, tRNA aminoacylation DNA maintenance and repair, iron-sulfur biogenesis, and iron homeostasis. We validate some of these findings and show that the *grx3* mutant is hypersensitive to oxidative, nitrosative and genotoxic stresses and shows decreased translational efficiency compared to wild-type. Finally, we show the *grx3* mutant displays decreased virulence in a disseminated infection model. Therefore, we propose *C. albicans* Grx3 is a global iron sensor critical for both the regulation of the iron homeostasis circuit as well as the functions of iron-sulfur cluster containing proteins involved in a wide array of diverse biological processes.

CHAPTER 1

Introduction

Candida albicans: an opportunistic pathogen of humans

Candida albicans is an exceedingly common opportunistic fungal pathogen. It can be found in the mouth, gastrointestinal and urogenital tracts, and skin of its human hosts. In the healthy, *C. albicans* poses no risk and generally lives a commensal lifestyle. However, in the immunocompromised *C. albicans* can become pathogenic and result in serious infection, with a mortality rate of up to 40% in the case of disseminated systemic infections [1]. In fact, *Candida* infections are one of the most common hospital acquired infections, and antifungal resistance remains a consistent problem and a critical area of ongoing research [2]. One aspect of *C. albicans* biology that allows it to occupy such distinct and diverse host niches is its ability to reversibly transition between several different morphological forms. These forms can be typified by differences in cell size, shape, gene expression, and even metabolism, with certain morphological forms better suited to particular host niches. Life in the mammalian host provides a constant struggle, and epigenetic regulation provides one mechanism to respond to the challenges encountered in the host.

White-opaque switching in *C. albicans*

One type of morphological transition available to *C. albicans* is the reversible,

epigenetic, and stochastic switch between the white and opaque yeast phases. Cells can switch spontaneously from the white, sometimes considered default, state to the opaque phase about once out of every 10^4 generations, depending on the strain [3]. In contrast to white colonies, which are dense, shiny white, and dome shaped, opaque colonies can be differentiated by their dull grey appearance and their proclivity to form flat, diffused colonies. Opaque cells also display a large elongated cell shape compared to their white counterparts, with a large vacuole and a pimples cell surface. Beyond the external differences between the white and opaque phases, the two cell types show differential regulation of hundreds of genes including those involved in mating, surface adhesion, cell surface composition, stress response, metabolism, and virulence [4, 5]. While opaque cells have reduced virulence compared to white cells in murine systemic infection models, they are better suited to colonizing the skin and show increased fitness in cutaneous infections, likely due to opaque cells' upregulation of secreted aspartyl proteases and other adhesion genes [6, 7]. White-opaque switching has also been suggested as a mechanism of host immune evasion, as opaque cells are less efficiently phagocytosed than white cells [8, 9].

The opaque phase can be induced by a variety of stimuli including the carbon source N-acetylglucosamine (GlcNAc) [10], high CO_2 [11], hypoxia [12], temperature [3, 13], and genotoxic stresses [14-16]. Conversely, growth at 37°C converts opaque cells *en masse* back to the white phase [3]. The opaque phase is considered the mating competent form of *C. albicans*, and opaque cells mate with 10^6 greater efficiency than white cells [17]. Opaque switching is inhibited through the actions of the *MTL*-encoded

$\alpha 1$ - $\alpha 2$ repressor complex and as a result, canonical opaque switching only occurs in cells that are homozygous at the mating locus (MTL), either **a/a** or α/α [17, 18]. However, despite the importance of the opaque phase in *C. albicans* biology, most clinical isolates are *MTLa/a* [17]. Recently, several labs have shown that opaque switching can occur in strains that are *MTLa/a*: *in vitro* by a combination of high CO₂, GlcNAc, and growth at 37°C, and *in vivo* by passage through the mammalian gut [19, 20]. Opaque cells derived by the latter method, termed GUT (gastrointestinally induced transition) cells, were distinct from typically derived *MTL* homozygous opaque cells, and showed enhanced commensalism compared to their white counterparts in gastrointestinal model [20].

Transcriptional regulation of white-opaque switching

The opaque state is induced through the expression of the master regulator Wor1 (White Opaque Regulator 1). Wor1 is expressed exclusively in opaque cells and forms an autoregulatory feedback loop by binding to its own promoter and inducing its own expression [21-23]. Opaque switching is also regulated at the levels of *WOR1* promoter chromatin and translation of *WOR1* transcripts. Hyperacetylation is associated with opaque induction and chromatin remodeling mutants alter switching rates [15, 16, 24-26]. Additionally, translation of *WOR1* transcript is another aspect subject to regulation, specifically through its unusually long 5'UTR region [27].

Wor1 is one member of a vast underlying transcriptional network regulating white-opaque switching. Additional transcriptional regulators involved in the white-

opaque regulatory circuit include Efg1, Ahr1, Czf1, Wor2, and Wor3 [21, 28-31].

Interestingly, although several of these transcription factors show differential expression in white and opaque phases, all of the regulators show similar enriched binding along each other's promoter regions [28]. One key region that exemplifies this type of regulation is the 8kb long *WOR1* promoter. Although *WOR1* expression is essential for opaque formation and maintenance, both positive and negative regulators of the opaque phase show highly enriched binding along the full length of the *WOR1* promoter in opaque cells. For example, the transcription factor Efg1, which is essential for maintenance of the white phase and occupies the *WOR1* promoter to repress *WOR1* expression in white cells, is downregulated four-fold in opaque cells yet shows three-fold enriched binding along the *WOR1* promoter [28]. Why both positive and negative regulators of *WOR1* expression occupy its active promoter in opaque cells remains unknown.

Wor1, a conserved fungal protein

While much work has been done to understand the transcriptional network underpinning white-opaque switching, less is known about the master regulator Wor1. Wor1 is a member of a conserved family of fungal proteins such as the transcriptional regulators Mit1 in *S. cerevisiae*, Ryp1 in *H. capsulatum*, and Sge1 in *F. oxysporum* [32-35]. These transcription factors are all involved in the regulation of developmental and lifestyle programs in their respective species including filamentous growth programs and virulence. This family of proteins shows no consensus in the C-terminal region but does

show conservation in the N-terminus, which contains two highly conserved domains termed WOPRa and WOPRb, both of which are essential for the DNA binding properties of this class of protein [33]. Recently resolved crystal structures of Wor1 have revealed that both WOPRa and WOPRb are structurally interwound together to form a compact globular domain that associates with DNA in a unique way, through simultaneous interactions with both the major and minor grooves [29, 36]. A conserved loop is essential for the recognition and sequence-specific binding of Wor1 to a 6bp motif, and point mutations of amino acids required for binding to this motif completely disrupts DNA association and consequently the ability of Wor1 to induce the opaque phase. In order to generate the crystals required for these experiments, the C-terminal region, as well as a linker portion between the two WOPR domains, had to be removed [36]. Indeed, not much is known about the structure or function of the C-terminus of Wor1. *In silico* analysis of the Wor1 amino acid sequence can find no known structures, motifs, or domains beyond the DNA-binding properties of the N-terminus. So while much work has been done to characterize the transcriptional regulation of *WOR1* and the rest of the white-opaque circuit in controlling opaque switching, very little is known about *how* Wor1 functions to induce and maintain the opaque phase. We seek to address this question in Chapter 3.

Life in the host: iron acquisition

As a host-dwelling organism, *C. albicans* must acquire essential nutrients from its surrounding host environment. This includes carbon sources for growth as well as

essential molecules and cofactors such as iron. As a ubiquitous cofactor, iron is essential to many processes including metabolism, respiration, DNA synthesis and repair, and more. In humans, most available iron is sequestered either as hemoglobin, transferrin, lactoferrin, or as ferritin [37, 38]. The purpose of this sequestration is two-fold for the host. Firstly, high iron levels can be toxic and lead to the formation of reactive oxygen species via the Fenton reaction; and secondly, as bacterial and fungal pathogens such as *C. albicans* are dependent on host iron to survive, sequestration by the host provides a mechanism of nutritional immunity and protection against pathogens [39, 40]. Studies have shown that individuals deficient in proper iron sequestration are more prone to infection by *C. albicans* and other pathogens [41].

To combat host-mediated sequestration of iron, *C. albicans* utilizes several different mechanisms to acquire iron from different sources within the host.

Hemoglobin uptake

Roughly 66% of iron in the human host is sequestered in the form of hemoglobin, the molecule responsible for carrying oxygen from the lungs throughout the body and CO₂ back to the lungs [42]. In order to exploit host hemoglobin as a source of iron, *C. albicans* releases a hemolytic enzyme resulting in erythrocyte lysis and release of free hemoglobin. The hemoglobin molecules are recognized by the *C. albicans* receptor Rbt5, resulting in hemoglobin endocytosis and hydrolysis through the activity of the hemoglobin oxygenase Hmx1, releasing ferrous iron (Fe²⁺) for use by *C. albicans*.

Transferrin reduction and uptake

The iron-binding glycoprotein transferrin (and to a lesser degree, lactoferrin) provides another mechanism of iron sequestration in the host. Although a very low percentage of the total iron pool in the human body is carried in the form of transferrin (~0.1%), it is the most important method of iron delivery [42]. *C. albicans* binds to transferrin through an unidentified receptor at the *C. albicans* cell surface. Once recognized, it is hypothesized that acidification of the extracellular space triggers the release of ferric iron (Fe^{3+}) from the transferrin molecule [43, 44]. The released ferric iron is then reduced to ferrous iron (Fe^{2+}) through the actions of the membrane-bound iron reductase Fre10. Ferrous iron is subsequently oxidized and imported into the cell through the coupled actions of the multicopper ferroxidase Fet34 and the iron permease Ftr1 [45].

Ferritin

Ferritin is a highly stable iron storage protein that displays the remarkable quality of being able to bind up to 4500 iron atoms in one protein nanocage [46]. *C. albicans* recognizes ferritin through the hyphal-expressed cell surface receptor Als3. Once bound, in a mechanism similar to that of transferrin, extracellular acidification encourages the dissociation of ferric iron from the ferritin particle. The free ferric iron is then reduced by Fre10 to ferrous iron, and oxidized and imported by the Fet34/Ftr1 ferroxidase/permease complex as in the case of transferrin [47].

Siderophore uptake

The final identified mechanism by which *C. albicans* is known to acquire iron from its host environment is through use of siderophores. Siderophores are secreted iron chelators that bind free iron with a higher affinity than host iron binding molecules, allowing microbial siderophores to strip iron from host proteins such as transferrin. Siderophore-mediated iron stripping was shown to be less efficient with ferritin, suggesting its inherent stability [48]. Interestingly, although *C. albicans* has been shown to display siderophore activity, no known siderophore biosynthetic genes have been identified [49]. However, *C. albicans* has also been shown to utilize siderophores produced by other microbial species in a mechanism termed 'iron parasitism' [37]. Once the siderophore has bound iron, it is recognized by the siderophore transporter Sit1 and imported into the cell [37, 38].

Regulation of iron homeostasis

Since *C. albicans* can inhabit a wide range of diverse host niches, the amount of bioavailable iron *C. albicans* encounters in the host can vary greatly depending on infection/colonization site. For example, free iron level is low in the bloodstream where it is mostly sequestered in the form of hemoglobin, but high in the gastrointestinal tract through host dietary consumption [38, 50]. As a natural inhabitant of both these host environments, *C. albicans* needs to be able to adapt to the environment it finds itself. While most efforts usually tend towards the acquisition of host iron, an overabundance of iron can be just as problematic, as excess iron can become toxic through the

generation of free radicals [39]. To combat this, *C. albicans* and other fungi have developed a conserved iron homeostasis transcriptional network to control iron uptake and utilization in response to iron level. In iron-replete conditions, the GATA family transcription factor Sfu1 represses the expression of iron uptake genes, making sure the intracellular iron level does not reach toxic levels [51]. Indeed, an *sfu1* mutant is sensitive on ferrochrome-supplemented plates and shows decreased commensalism in a murine gastrointestinal model where iron levels are high, confirming that Sfu1 is critical for *C. albicans* response to excess iron [51]. Sfu1 also directly represses the expression of the Cys₆Zn₂ transcription factor Sef1, which functions as an activator of iron uptake gene expression. Sef1 also positively regulates the expression of the third member of the *C. albicans* iron homeostasis regulon, the CCAAT binding protein (CBP) transcription factor [51]. Hap43 represses the expression of nonessential iron utilization genes as well as the expression of *SFU1* [51]. Together, Sef1 and Hap43 ensure that the intracellular iron pool is sufficient for necessary biological processes to function. Therefore, Sef1 and Hap43 are essential for *C. albicans* response to iron limitation. In confirmation of this, loss of either *sef1* or *hap43* results in an extreme sensitivity to low-iron media as well and decreased virulence of the *sef1* mutant in a murine systemic infection model [51].

Both Sfu1 and Hap43 are highly conserved proteins with conserved roles in the regulation of iron homeostasis in a broad range of fungi including *S. pombe*, *C. neoformans*, and *H. capsulatum* [52-56]. However, *C. albicans* is unique in its incorporation of Sef1 into the iron regulon, and consequently, less is known about how

Sef1 contributes to this regulation and how Sef1 itself is regulated [51, 57, 58]. One question that remains unanswered, however, is how iron level is sensed and integrated into the regulation of the iron homeostasis circuit in *C. albicans*.

Glutaredoxins and iron homeostasis

One potential mechanism of iron sensing is through the actions of the glutaredoxin family. Glutaredoxins (Grxs) are a highly conserved family of proteins that can be found in all kingdoms of life. Similar to thioredoxins (Trxs), glutaredoxins are a family of thiol-disulfide oxidoreductases that are critical to the redox homeostasis of the cell [59-61]. Cysteines become irreversibly oxidized in the presence of reactive oxygen species (ROS) leading to protein inactivation. Grxs can combat this process in two ways: (1) by reducing the oxidized cysteine back to their original forms and (2) by protecting vulnerable cysteines via reversible glutathionylation [60, 62]. Additionally, glutathionylation of target thiols can also serve as a regulatory modification that alters protein function [60, 63].

Grxs fall into two broad categories based off their active site motifs. Class I is comprised of dithiol Grxs, which contain two cysteines in their active site and the consensus sequence CPF/YC [59]. Class II consists of the monothiol glutaredoxins, characterized by their CGFS active site motif with a single cysteine [61]. Monothiol Grxs can further be subdivided into single domain, containing a single Grx domain, and multi domain, containing both Trx and Grx domains [60]. Additionally, many monothiol domains also carry a mitochondrial targeting sequence, directing their localization to the

mitochondria. Both monothiol and dithiol glutaredoxins use glutathione and NADPH to in redox reactions, although by different mechanisms [60].

Interestingly, although expected to possess the ability to do so, reconstitution assays have shown that monothiol Grxs show little if any oxidoreductase *in vivo* [59, 61, 64]. Instead, monothiol Grxs have been shown to be important in the biogenesis and trafficking of iron-sulfur (Fe-S) clusters, cofactors critical for a multitude of biological processes [59-61]. Fe-S clusters are synthesized in the mitochondria through the actions of the Fe-S cluster assembly (ISC) system, in which the monothiol glutaredoxin Grx5 plays a critical role in the second step of ISC assembly as a downstream Fe-S cluster transfer protein [65]. Defects in mitochondrial Fe-S cluster synthesis have far reaching consequences, with associated Fe-S diseases including Friedreich ataxia, anemia, and dysfunctional erythropoiesis [66]. It has been shown that dithiol Grxs have also possess the capacity to bind Fe-S clusters, however it is believed that their predominant function is in maintaining redox homeostasis [59]. Consistent with their observed roles in Fe-S cluster biogenesis and transfer, monothiol Grxs have been implicated in the regulation of iron regulons in fungi, plants, and mammals [59-61]. In *S. cerevisiae*, under iron sufficiency the monothiol cytosolic Grx3/4 forms and Fe-S cluster bridge and interacts with the BoIA-like proteins Fra1 and Fra2 [67]. This complex binds and sequesters the iron regulatory transcription factor Aft1/2 in the cytosol, prevent its nuclear localization and induction of iron uptake genes [67]. Under iron limitation, low Fe-S cluster level leads results in the loss of the Grx-Fra-Aft complex formation and Aft1/2 translocates to the nucleus and induces the expression of iron uptake genes [67].

In *S. pombe*, Grx4 contributes to the iron homeostasis circuit on two levels. Firstly, Grx4 controls the activity of the Sfu1 homolog Fep1. Under iron replete conditions, Grx4 and Fep1 repress iron uptake gene expression directly through occupancy at target promoters. Similar to the role of Sfu1 on Sef1 and Hap43 regulation, Fep1 also represses the expression of the Hap43 homolog Php4 [68]. Under iron limitation Grx4 does not carry an Fe-S cluster due to low iron content but still interacts with Fep1 [52, 69]. Fep1 then donates its own bound Fe molecule to Grx4 in a reverse transfer mechanism that results in Fep1 dissociation from target promoters [52]. The second mechanism of Grx4-mediated regulation of the iron homeostasis circuit involves Grx4-mediated cytosolic sequestration of Php4 in iron replete conditions; tethered in the cytosol, Php4 is rendered inactive [70]. However, in low iron Php4 is released from Grx4 and translocates to the nucleus where it can repress both Fep1 and iron utilization gene expression [70]. Whether Grx3-mediated regulation of the iron homeostasis circuit is conserved in *C. albicans* is explored in Chapter 4.

***C. albicans* biology: a proteomic approach**

Much of the *C. albicans* field has focused on genetic approaches to understanding biological phenomena. While functional genetics is an extremely powerful tool, phenotypes can be missed if not assayed under the proper/specific conditions. Proteomic approaches such as identification of protein interactions can provide complementary data to supplement an observed genetic phenotype. Therefore, a multi-pronged approach that utilizes both functional genetics and proteomic data would

provide unique insights into biologically relevant questions in the *C. albicans* field.

Presented in this dissertation, we have attempted to address two overarching questions regarding *C. albicans* biology. In chapter 3, we assess the master regulator of the opaque phase Wor1 and how it functions to induce and maintain the opaque phase through regulation of the general co-repressor Tup1. In chapter 4, we examine the role of the cytosolic monothiol glutaredoxin Grx3 in the regulation of the iron homeostasis circuit and identify new pathways and processes dependent on Grx3 for proper function.

CHAPTER 2

Materials and Methods

Strains and culturing conditions

Strains were grown in YEP (1% yeast extract, 2% peptone), YNB medium (0.17% Difco yeast nitrogen base w/o ammonium sulfate, 0.5% ammonium sulfate), or synthetic complete (SC) medium (0.17% Difco yeast nitrogen base w/o ammonium sulfate, 0.5% ammonium sulfate, complete supplement amino acid mixture) plus 2% either dextrose or maltose as indicated. Iron depletion was induced by growth in YPD media containing 300 μ M or 500 μ M bathophenanthroline disulfonic acid (BPS), as indicated. High iron stress was induced through addition of 100 μ M FeCl₃. Cells grown for the purpose of HBH purification were grown in media supplemented with 4 μ M biotin. *pMET3-TUP1* cells were routinely cultured in SC media lacking methionine, and *pMET*-mediated repression was induced by addition of 5mM methionine. White and opaque cells were maintained at 30°C and room temperature, respectively.

Plasmid and strain construction

For Chapter 3:

***pMAL2-WOR1-HBH* plasmid.** The plasmid pFA6a-HTB-TRP1 was graciously provided by Dr. Kaiser [71]. Flanking MluI and KpnI sites were added to the 5' and 3' ends of the construct, respectively, as well as an additional 6xHis tag to the C-terminal end. The

construct was codon-optimized for *C. albicans* (conversion of serine-coding CTGs to *Candida* leucines) using a two-PCR process with long primers (primers 1-4 listed in S3 Table) carrying point mutations, and an additional KpnI site was removed using the same process. The final product was digested with MluI and KpnI, and inserted into a MluI and KpnI digested pMAL2-WOR1-carrying plasmid (p905) generated from the BES119 vector backbone [72], to yield the final construct *pMAL2-WOR1-HBH* (p1251). The plasmid was digested with AscI for integration at the *ADE2* locus and transformed into *MTLa/a* wild-type strain JYC5, generating HLY4532[73].

***pMAL2-TUP1-HBH* plasmid.** *TUP1* was amplified with primers 5 and 6 adding flanking XbaI and MluI restriction sites to the 5' and 3' ends, respectively. The sequence was amplified by PCR, double digested with XbaI and MluI. The *pMAL2-WOR1-HBH* backbone was similarly digested to remove the *WOR1* sequence, purified through gel extraction, and *TUP1* was inserted to become *pMAL2-TUP1-HBH* (p1101). The plasmid was digested with either AscI for integration at *ADE2* (HLY4537) or BglII for integration at *TUP1* and transformed into JYC5, generating HLY4542 [73].

***pMET3-TUP1-HIS3* strain.** The *HIS1* gene (for selection) and *MET3* promoter were amplified and fused together by PCR (primers 7-10), as described [74]. Flanking sequences of around 250bp of the *TUP1* 5' upstream region and around 30bp of the start of the *TUP1* gene were also added by PCR to the *HIS1-pMET3* construct. The final PCR product (*5'TUP1-HIS1-pMET3-TUP1*) was transformed into the haploid strain GZY822 (graciously provided by Dr. Berman [75]) and verified for integration.

***pMET3-TUP1 pWOR1-GFP* strain.** The plasmid pWOR1-GFP[21], which contains 3.2kb of the *WOR1* promoter, was digested with AscI for integration into the *ADE2* locus

and transformed into the above *pMET3-TUP1* strain (HLY4533) to obtain the haploid HLY4535.

***pMET3-TUP1 wor1::ARG4* strain:** *WOR1* was deleted with *ARG4* in the *pMET3-TUP1* conditional mutant strain (HLY4533) using the method described (primers 13-16) [74], with *ARG4* integrated into the *WOR1* coding sequence to generate the haploid HLY4539.

***tup1 wor1* strain:** *TUP1* was deleted in a previously published diploid *MTLa/a wor1* mutant strain (HLY3555) by CRISPR-Cas9 editing as described to generate HLY4540 [21, 76]. Primers 23 and 24 were used to generate the *TUP1* sgRNA plasmid, and primers 25 and 26 were used for the repair template.

***pTUP1-TUP1-3xHA* strain:** The 3xHA sequence was amplified by PCR with primers 11 and 12 to replace the HBH tag in the *pMAL2-TUP1-HBH* (p1101) plasmid using the *MluI* and *KpnI* sites, resulting in the final construct, *pMAL2-TUP1-3xHA* (p1142). The resulting plasmid was linearized with *BglII* for integration into the *TUP1* locus of the wild-type *MTLa/a* strain JYC1 to obtain the diploid strain, HLY4538.

***pTUP1-TUP1-3xHA pMAL2-WOR1-FLAG* strain:** The *URA3* marker was replaced with *HIS1* in the *pMAL2-TUP1-HBH* vector (p1143) using *BamHI* and *NotI*. The plasmid was then digested with *NotI* and *KpnI* to remove the *TUP1* coding sequence and replace it with the *WOR1* coding sequence to create the *HIS1*-carrying plasmid, *pMAL2-WOR1-HBH* (p1144). A 3xFLAG tag was then added to the C-terminal region of the *WOR1* coding sequence using PCR with a long primer (primers 21 and 22). The resulting amplicon was digested with *XbaI* and *KpnI* and swapped in place of *WOR1-HBH*. The resulting plasmid, *pMAL2-WOR1-FLAG* (p1162), was digested with *HpaI* and

transformed into the *pTUP1-TUP1-3xHA* strain (HLY4541) and integrated into the *WOR1* locus.

***pMAL2-WOR1* strain:** the plasmid *pMAL2-WOR1* (p880) was digested with *Ascl* and integrated into the *ADE2* locus of the diploid *MTLa/α* strain CAI4 to obtain HLY4543 [21, 77].

For Chapter 4:

***grx2*, *grx3*, and *grx5* strains:** Strains were taken from the GRACE library and loss of the tetracycline transactivator was selected for by growth on 5-FOA plates [78].

***grx1* strain:** *grx1* was deleted from the CaSS1 wild-type strain (HLY4494) by CRISPR-Cas9 editing as described [76] to generate the strain HLY4490. Primers 62 and 63 were used to generate the *GRX1* sgRNA plasmid, and primers 64 and 65 were used for the repair template.

***sfu1 grx3* strain:** *grx3* was deleted from the *sfu1* mutant strain (Homann [79]) by CRISPR-Cas9 editing to generate the strain HLY4510. Primers 66 and 67 were used to generate the *GRX3* sgRNA plasmid, and primers 68 and 69 were used for the repair template.

***pMAL2-SFU1-13xMYC* plasmid:** *SFU1* was amplified by primers 70 and 71. The resulting amplicon was inserted by Gibson assembly into an *XbaI* and *PacI* digested *pMAL2-WOR1-13xMYC* plasmid (p905) previously generated from the BES119 vector backbone. The resulting plasmid was digested with *Ascl* for insertion into the *ADE2* locus and transformed into the CaSS1 wild-type strain resulting in HLY4498 or into the *grx3* mutant strain resulting in HLY4507.

pMAL2-SEF1-GFP plasmid: *SEF1* was amplified with primers 72 and 73, and the resulting amplicon was inserted by Gibson assembly into an XbaI and MluI digested pMAL2-MYC-GFP plasmid (p866). The resulting plasmid was digested with AscI for insertion into the *ADE2* locus or with AccI for insertion into the *SEF1* locus.

pGRX3-GRX3-FLAG plasmid: The *GRX3* coding sequence and promoter were amplified using primers 74 and 75. The FLAG sequence was amplified from the pMAL2-WOR1-FLAG plasmid (p1142) [80] using primers 76 and 77. The final pGRX3-GRX3-FLAG product was inserted by Gibson assembly into a PstI and KpnI digested pWOR1-GFP plasmid (p1029) carrying the NAT resistance gene. The resulting plasmid was digested with HindIII for insertion into the *pGRX3* locus.

pMAL2-GRX3-HBH plasmid: *GRX3* was amplified using primers 78 and 79. The *GRX3* amplicon were inserted by Gibson assembly into an XbaI and MluI digested pMAL2-WOR1-HBH plasmid (p1251) [80]. The resulting plasmid was digested with AscI for insertion into the *ADE2* locus and transformed into the *grx3* mutant, generating the strain HLY4559.

HBH purification for mass spectrometry

Protein purification was performed as described by Tagwerker et al., with modifications[71]. 200ml cultures of cells expressing either the Wor1-HBH, Grx3-HBH, or the untransformed background strain were grown in YPM at room temperature, cross-linked for 10 min by the addition of formaldehyde to a final concentration of 1%, centrifuged at 1,892 x g for 5min, washed twice with H₂O, and lysed with Buffer A (8M urea, 300mM NaCl, 0.5% NP-40, 50mM sodium phosphate pH 8, 50mM Tris pH 8,

20mM imidazole, 1mM PMSF, 1 Roche Complete EDTA-free protease inhibitor tablet) by five pulses of 20 sec intervals using glass beads and the FastPrep system (setting 5.0). Lysates were collected and centrifuged for 10 min at 16,000 x g. In one experiment an optional sonication step to shear DNA and help extract chromatin-associated protein complexes was incorporated at this point using the same protocol described in the ChIP methods section. Protein concentration was measured and the appropriate volume of Ni²⁺-Sephacrose (GE Healthcare) was added to the clarified lysates. Samples and beads were incubated for 6h, then washed with 5-10x bead bed volume twice with Buffer A, twice with Buffer B (8M urea, 300mM NaCl, 0.5% NP-40, 50mM sodium phosphate, 50mM Tris, final pH 6.3), and eluted twice with 2.5x bead bed volume of Buffer C (8M urea, 200mM NaCl, 50mM sodium phosphate, 10mM Tris, 2% SDS 10mM EDTA, 300mM imidazole, final pH 4.3). Eluate pH was neutralized by addition of 10% eluate volume of 1M Tris pH 8, and samples were incubated overnight with streptavidin-conjugated agarose beads (Thermo) pre-washed in Buffer D (8M urea, 200mM NaCl, 100mM Tris pH 8, 2% SDS). Samples were then washed with 5-10x bead bed volume twice with Buffer D, twice with Buffer E (8M urea, 200mM NaCl, 100mM Tris pH 8, 0.2% SDS), twice with Buffer F (8M urea, 200mM NaCl, 100mM Tris pH8), and five times with 50mM NH₄HCO₃. At all steps, samples were collected and saved for Western blot verification of successful purification. After final washes, samples were trypsin digested overnight at 37°C. Formic acid was added to a final concentration of 1% to stop the digestion and the supernatant was collected. Beads were washed three times with half the bead volume of 25% acetonitrile and 0.1% formic acid, and the supernatants were collected and pooled each time. Samples were concentrated with a SpeedVac and

washed twice with 100µl H₂O before final resuspension in 10µl H₂O and analysis by mass spectrometry.

Mass Spectrometry and Data Analysis

LC-MS/MS analysis was carried out for peptide samples using an EASY NanoLC 1000 system (ThermoFisher Scientific) coupled on-line with an LTQ-Orbitrap XL MS (ThermoFisher Scientific) as described[81]. Each cycle of an MS/MS experiment includes one MS scan in FT mode (350-1400 m/z, resolution of 60,000 at m/z 400) followed by data-dependent MS2 scans in the LTQ with normalized collision energy at 35% on the top ten peaks. Raw data were searched against a database consisting of Candida proteins (PA.orf_trans_all_assembly_21_2009_0306_v2 with a total of 6243 protein entries) using MaxQUANT. The mass tolerance for parent ions and fragment ions were set as ± 20 ppm and 0.5 Da, respectively. Trypsin was set as the enzyme, and a maximum of two missed cleavages were allowed. Protein N-terminal acetylation and methionine oxidation were selected as variable modifications. Protein and PSM FDR was set as 0.01. LFQ and iBAQ protein quantitation were calculated using a minimum ratio count of 2 peptides[82, 83].

Tup1 depletion and switching assays

pMET-TUP1 haploid cells were cultured overnight in SCD media lacking methionine to maintain *TUP1* expression. Cells were then washed and used to inoculate two fresh 50 ml cultures, one lacking methionine and the other supplemented with 5mM methionine. Cultures were grown at room temperature over the course of several days, and samples

were taken at the time points indicated. Samples saved for qPCR analysis were spun down and flash frozen. Samples for switching assays were washed three times with H₂O before diluting and plating onto SCD met– plates. Plates were grown up at room temperature for 5-7 days and colonies were counted to determine white-opaque percentages.

Opaque stability assays

Opaque cells were grown overnight at room temperature in SCD. Cells were diluted into fresh SC media with 2% of the indicated carbon source, cultured for 3 hours, then shifted to 37°C for 24hr. Cells were then plated onto SCD plates, grown for 5-7 days at room temperature, and scored for percent opaque. Opaque CAI4 carrying *pMAL2-WOR1* were grown overnight in SCM, washed three times with H₂O, and either plated onto SC plates of the indicated carbon source and incubated at room temperature or 37°C for 5-7 days and scored, or were inoculated into liquid SC media with the indicated carbon source, cultured for 3 hours at room temperature, then shifted to 37°C. After 24hr, cells from liquid cultures were then spread onto SCD plates and grown at room temperature for 5-7 days then scored. Whole and sector opaque colonies were counted.

Western blotting

Cells were collected by centrifugation and lysed with glass beads using a FastPrep at a setting of 5.0 with five pulses of 20 sec intervals. Lysates were clarified by centrifugation at 16,000 x g for 10min at 4°C. Samples were prepared with sample buffer and loaded

onto 8% SDS-PAGE gels, transferred to nitrocellulose membranes, and probed for proteins of interest. HBH was detected by a mouse monoclonal antibody against the RGS6H sequence (Qiagen), 13xMyc with a rabbit polyclonal (Santa Cruz), PSTAIRE (Cdc28) with a rabbit polyclonal (Santa Cruz), HA with an HRP-conjugated anti-HA antibody (Roche), and FLAG with a mouse monoclonal (Sigma). All non-HRP-conjugated antibodies were detected by either goat anti-mouse or goat anti-rabbit HRP-conjugated secondary antibodies (Bio-Rad).

Quantitative RT-PCR

RNA samples were prepared using Qiagen's RNeasy and On-Column DNase Digestion kits. cDNA was synthesized using a Bio-Rad iScript Reverse Transcription Kit and 1 μ g total RNA. Quantitative PCR was performed using Bio-Rad SYBR Green mix on a Bio-Rad iCycler. Primers used are listed in S3 Table.

Spot testing

Overnight cultures of the indicated strains were washed three times with H₂O and diluted to an OD₆₀₀ of 1.0. Strains were then serially diluted 10-fold four times, and 2.5-5 μ L were spotted onto plates of the indicated media. Plates were grown for 2-3 days at 30°C. For low iron stress, strains were spotted onto YPD plates containing 300 μ M or 500 μ M bathophenanthroline disulfonic acid (BPS) as indicated. For high iron stress, strains were spotted onto YPD plates with 100 μ M FeCl₃. For oxidative stress, cells were treated to 3 or 5mM H₂O₂. Genotoxic stress was induced through treatment with

30mM hydroxyurea (HU) or 0.03% methyl methanesulfonate (MMS), and nitrosative stress induced with 2.5mM or 5mM dipropyleneetriamine (DPTA) NONOate.

Chromatin Immunoprecipitation

ChIP was performed as described, with slight modifications[84]. For Chapter 3, overnight cultures grown at room temperature were diluted to an OD₆₀₀ of 0.1 and grown to OD₆₀₀ 1.0. Cells grown in different carbon sources were pre-cultured in medium containing the indicated carbon source before dilution. For Chapter 4, strains were cultured overnight in YPM at 30°C then diluted into fresh YPM with or without 500µM BPS and grown for 5hr. Samples were then fixed with 1% formaldehyde. DNA was sheared by sonication for 6 cycles of 20s at 40s intervals using a Bioruptor (Diagenode) and 1% input samples were saved. Experiments examining multiple regulator binding events were prepared from the same chromatin samples and split prior to immunoprecipitation. The HA-tag was immunoprecipitated using a 12CA5 anti-HA mouse monoclonal antibody (Roche), and the FLAG-tag was immunoprecipitated using anti-FLAG M2 mouse monoclonal antibody (F3165, Sigma). DNA from input and IP samples was quantified by qPCR. Enrichment of tagged samples was quantified by comparing the region of interest IP (bound/input) over the control region *ADE2* IP (bound/input) and further normalizing to an untagged strain, with values representing at least three independent experiments and error bars representing the s.d. Primers used are listed in S3 Table.

Immunoprecipitation

Cultures were grown overnight at 30°C in YPM then diluted into fresh YPM in the presence or absence of 500µM BPS. Cultures were grown for another 5hr then harvested and lysed. Clarified lysates were incubated at 4°C with 50µL pre-conjugated FLAG M2 beads (Sigma) for 2hr, washed five times with lysis buffer, and eluted in TE buffer with 1% SDS. Samples were resolved by SDS-PAGE and analyzed by Western blot.

Flow cytometry

Flow cytometry was performed using the BD FACSCalibur system. Cells were passed through cell-strainer caps (BD Falcon) and sonicated prior to flow cytometry analysis. 10,000 cells were counted per sample and measured for GFP expression using the FL1-H channel. FACS data was analyzed using BD CellQuest Pro software.

Microscopy

For Chapter 3, haploid cells carrying *WOR1* promoter driven GFP (HLY4535) were grown in SCD medium with or without methionine at room temperature for 48hr. For Chapter 4, cultures were grown overnight in YPD then diluted into fresh YPD or YPM (for *pMAL2-SEF1-GFP*) containing 500µM BPS, cultured for 6hr, then imaged. Cells were stained with DAPI (4', 6-diamidino-2-phenylindole) for nuclear localization. Images were taken using an inverted Zeiss Axio Observer.Z1 microscope (Carl Zeiss MicroImaging) equipped with an X-Cite series 120 mercury lamp. DAPI, GFP fluorescence, and cell morphology were imaged using the DAPI, GFP, and DIC channels, respectively.

Nascent protein labeling

Wild-type and *grx3* cells were grown overnight in Met- media, then treated for 1hr at 30°C with either 35mM L-azidohomoalanine (AHA) (Invitrogen) or methionine as a control. Protein was extracted and 100ug total protein was prepared using the Click-iT Protein Reaction Buffer kit (Invitrogen) and subsequently labeled with tetramethylrhodamine (TAMRA) (Invitrogen). Labeled nascent proteins were separated on SDS-PAGE and imaged using a Fujifilm LAS-4000 Imager (Fujifilm) with an excitation of 550nm and emission of 570nm. Finally, the gel was stained by coomassie to verify equal loading.

Virulence studies

Virulence assays were performed previously as described [85]. Briefly, five mice per indicated strain were injected with *C. albicans* cells into the lateral tail vein and monitored daily for survival.

Table 2.1: *C. albicans* strains used in Chapter 3

Strain	Description	Genotype	Source
JYC5	WT	<i>MTLa/a ura3Δ::imm434/ura3Δ::imm434</i>	Chen <i>et al.</i> , 2002
HLY4532	<i>pMAL2-WOR1-HBH</i>	<i>MTLa/a ura3Δ::imm434/ura3Δ::imm434 ADE2/ade2Δ::MAL2p-WOR1-HBH-URA3</i>	This study
HLY4538	<i>pMAL2-TUP1-3xHA</i>	<i>MTLa/a ura3Δ::imm434/ura3Δ::imm434 TUP1/tup1Δ::TUP1p-TUP1-3xHA-URA3</i>	This study
HLY4537	<i>pMAL2-TUP1-HBH</i>	<i>MTLa/a ura3Δ::imm434/ura3Δ::imm434 ADE2/ade2Δ::MAL2p-TUP1-HBH-URA3</i>	This study
HLY4542	<i>pTUP1-TUP1-HBH</i>	<i>MTLa/a ura3Δ::imm434/ura3Δ::imm434 TUP1/tup1Δ::MAL2p-TUP1-HBH-URA3</i>	This study
HLY4536	<i>pMAL2-TUP1-13xMYC</i>	<i>MTLa/a ura3Δ::imm434/ura3Δ::imm434 ADE2/ade2Δ::MAL2p-TUP1-13xMYC-URA3</i>	This study
HLY3555	<i>pWOR1-GFP</i>	<i>MTLa/a ura3Δ::imm434/ura3Δ::imm434 WOR1/wor1Δ::WOR1p-GFP-URA3</i>	Huang <i>et al.</i> , 2006
GZY822	WT, haploid	<i>MTLa his4 ura3Δ::HIS4 arg4Δ::FRT his1Δ::FRT</i>	Hickman <i>et al.</i> , 2013
HLY4533	<i>pMET3-TUP1</i>	<i>MTLa his4 ura3Δ::HIS4 arg4Δ::FRT his1Δ::FRT tup1Δ::MET3p-TUP1-HIS1</i>	This study
HLY4535	<i>pMET3-TUP1 + pWOR1-GFP</i>	<i>MTLa his4 ura3Δ::HIS4 arg4Δ::FRT his1Δ::FRT tup1Δ::MET3p-TUP1-HIS1 ade2Δ::WOR1p-GFP-URA3</i>	This study
HLY4539	<i>pMET3- TUP1 + wor1</i>	<i>MTLa his4 ura3Δ::HIS4 arg4Δ::FRT his1Δ::FRT tup1Δ::MET3p-TUP1-HIS1 wor1Δ::ARG4</i>	This study
HLY4534	<i>pWOR1-GFP</i>	<i>MTLa his4 ura3Δ::HIS4 arg4Δ::FRT his1Δ::FRT ade2Δ::WOR1p-GFP-URA3</i>	This study
JYC1	WT	<i>MTLa/a ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG</i>	Chen <i>et al.</i> , 2002
HLY4541	<i>pWOR1-WOR1-3XFLAG + p-TUP1-TUP1-3XHA</i>	<i>MTLa/a ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG WOR1/wor1Δ::MAL2p-WOR1-3xFLAG-HIS1 TUP1/tup1Δ::TUP1-3xHA-URA3</i>	This study
HLY3570	<i>wor1</i>	<i>MTLa/a ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG wor1Δ::ARG4/wor1Δ:: HIS1</i>	Huang <i>et al.</i> , 2006
HLY4540	<i>wor1 tup1</i>	<i>MTLa/a ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG wor1Δ::ARG4/wor1Δ::HIS1 NEUT5L/neut5LΔ::CaCAS9-sgRNA-TUP1 tup1/tup1</i>	This study
CAI4	WT	<i>MTLa/a ura3Δ::imm434/ura3Δ::imm434</i>	Fonzi <i>et al.</i> , 1993
HLY4543	<i>MTLa/a pMAL2-WOR1</i>	<i>MTLa/a ura3Δ::imm434/ura3Δ::imm434 ADE2/adeΔ::MAL2p-WOR1-URA3</i>	This study

Table 2.2: *C. albicans* strains used in Chapter 4

Strain	Description	Genotype	Source
CaSS1	WT	<i>ura3Δ::imm434/ ura3Δ::imm434</i> <i>his3Δ::hisG/his3Δ::hisG URA3 tet R-GAL4AD</i>	Roemer <i>et al.</i> , 2003
HLY4494	WT, ura -	<i>ura3Δ::imm434/ ura3Δ::imm434</i> <i>his3Δ::hisG/his3Δ::hisG</i>	This study*
HLY4490	<i>grx1</i>	<i>ura3Δ::imm434/ ura3Δ::imm434 grx1Δ/grx1Δ NAT</i> <i>CRISPR/Cas9</i>	This study
HLY4491	<i>grx2</i>	<i>ura3Δ::imm434/ ura3Δ::imm434</i> <i>his3Δ::hisG/his3Δ::hisG grx2Δ::HIS3/GRX2pΔ::Tetp-SAT1-1</i>	This study*
HLY4492	<i>grx3</i>	<i>ura3Δ::imm434/ ura3Δ::imm434</i> <i>his3Δ::hisG/his3Δ::hisG grx3Δ::HIS3/GRX3pΔ::Tetp-SAT1-1</i>	This study*
HLY4493	<i>grx5</i>	<i>ura3Δ::imm434/ ura3Δ::imm434</i> <i>his3Δ::hisG/his3Δ::hisG grx5Δ::HIS3/GRX5pΔ::Tetp-SAT1-1</i>	This study*
<i>sfu1</i>		<i>arg4Δ/ arg4Δ leu2Δ/leu2Δ his1Δ/his1Δ</i> <i>URA3/ura3Δ::imm434 IRO1/iro1Δ::imm434</i> <i>sfu1Δ::Candida dubliniensis HIS1/sfu1Δ::C. maltosa LEU2</i>	Homann <i>et al.</i> , 2009
<i>sef1</i>		<i>arg4Δ/ arg4Δ leu2Δ/leu2Δ his1Δ/his1Δ</i> <i>URA3/ura3Δ::imm434 IRO1/iro1Δ::imm434</i> <i>sef1Δ::Candida dubliniensis HIS1/sef1Δ::C. maltosa LEU2</i>	Homann <i>et al.</i> , 2009
<i>hap43</i>		<i>arg4Δ/ arg4Δ leu2Δ/leu2Δ his1Δ/his1Δ</i> <i>URA3/ura3Δ::imm434 IRO1/iro1Δ::imm434</i> <i>hap4Δ::Candida dubliniensis HIS1/hap4Δ::C. maltosa LEU2</i>	Homann <i>et al.</i> , 2009
HLY4510	<i>sfu1 grx3</i>	<i>arg4Δ/ arg4Δ leu2Δ/leu2Δ his1Δ/his1Δ</i> <i>URA3/ura3Δ::imm434 IRO1/iro1Δ::imm434</i> <i>sfu1Δ::Candida dubliniensis HIS1/sfu1Δ::C. maltosa LEU2 grx3Δ/grx3Δ NAT CRISPR/Cas9</i>	This study
HLY4498	pMAL2-SFU1-13MYC	<i>ura3Δ::imm434/ ura3Δ::imm434</i> <i>his3Δ::hisG/his3Δ::hisG ADE2/ade2Δ::MAL2p-SFU1-MYC-URA3</i>	This study
HLY4507	<i>grx3</i> + pMAL2-SFU1-13MYC	<i>ura3Δ::imm434/ ura3Δ::imm434</i> <i>his3Δ::hisG/his3Δ::hisG grx3Δ::HIS3/GRX3pΔ::Tetp-SAT1-1 ADE2/ade2Δ::MAL2p-SFU1-myc-URA3</i>	This study
HLY4500	pMAL2-SFU1-13MYC + pGRX3-GRX3-FLAG	<i>ura3Δ::imm434/ ura3Δ::imm434</i> <i>his3Δ::hisG/his3Δ::hisG ADE2/ade2Δ::MAL2p-SFU1-myc-URA3 GRX3/GRX3::Grx3p-GRX3-FLAG-NAT</i>	This study
HLY4560	WT + pMAL2-SEF1-GFP	<i>ura3Δ::imm434/ ura3Δ::imm434</i> <i>his3Δ::hisG/his3Δ::hisG ADE2/ade2Δ::MAL2p-SEF1-GFP-URA3</i>	This study
HLY4561	<i>grx3</i> + pMAL2-SEF1-GFP	<i>ura3Δ::imm434/ ura3Δ::imm434</i> <i>his3Δ::hisG/his3Δ::hisG grx3Δ::HIS3 ADE2/ade2Δ::MAL2p-SEF1-GFP-URA3/GRX3pΔ::Tetp-SAT1-1</i>	This Study
HLY4562	<i>sfu1</i> +	<i>arg4Δ/ arg4Δ leu2Δ/leu2Δ his1Δ/his1Δ</i>	This study

	pMAL2-SEF1-GFP	<i>URA3/ura3Δ::imm⁴³⁴ IRO1/iro1Δ::imm434 sfu1Δ::Candida dubliniensis HIS1/sfu1Δ::C. maltosa LEU2 ADE2/ade2Δ::MAL2p-SEF1-GFP-URA3</i>	
HLY4563	<i>sfu1 grx3 + pMAL2-SEF1-GFP</i>	<i>arg4Δ/ arg4Δ leu2Δ/leu2Δ his1Δ/his1Δ URA3/ura3Δ::imm434 IRO1/iro1Δ::imm434 sfu1Δ::Candida dubliniensis HIS1/sfu1Δ::C. maltosa LEU2 grx3Δ/grx3Δ NAT CRISPR/Cas9 ADE2/ade2Δ::MAL2p-SEF1-GFP-URA3</i>	This study
HLY4559	<i>grx3 + pMAL2-GRX3-HBH</i>	<i>ura3Δ::imm434/ ura3Δ::imm434 his3Δ::hisG/his3Δ::hisG grx3Δ::HIS3/GRX3pΔ::Tetp- SAT1-1 ADE2/ade2Δ::MAL2p-GRX3-HBH-URA3</i>	This Study

* Derived from Roemer *et al.*, 2009.

Table 2.3: Primers used in Chapter 3

Number	Direction	Sequence (5'-3')	Purpose
1	F	GCGACGCGTAGGGGTTACATCATCACCACCATC ATG	HBH generation
2	R	GTTTGACCAGCTTTAACAGTGTACCTTCTTTTAC CAATATCTTAGAAACAGTACCAGCAAGAGGGGCA GGGATTTC	
3	F	GTAAGAAGGTGACACTGTAAAGCTGGTCAAAC AGTTTTGGTGCTTGAGGCTATGAAATGGAGACA GAAATTAAC	
4	R	GGGGTACCCTTAATTAATCACTAATGATGGTGGTG ATGATGAACGCCGATCTTGATTAGACCTTGACCAC	
5	F	GCT CTAGA ATGTCCATGTATCCCCAACG	Insertion of <i>TUP1</i> into HBH vector (replace <i>WOR1</i>)
6	R	GCG ACGCGT TTTTTTGGTCCATTTCCAAATTC	
7	F	TTGGGCAGTGACAGCTTTAC	<i>pMET3-TUP1</i> generation
8	R	CACGGCGCGCCTAGCAGCGGGATGGAAGAAGTT GTGTTGT	
9	F	GTCAGCGCCGCATCCCTGCGCGCTGAAAACTA CGAACAATTGTC	
10	R	CTCTGTCAAACGTTGTTGGTGCTGGGTGCGTTGG GGATACATGGACATTGCTGGTGTTTCTGGGGAG GGTATTAC	
11	F	CG ACGCGT TACCCATACGATGTTC	HA amplification to swap into TUP1- HBH plasmid
12	R	GG GTACCT CACTAAGCAGCGTAATCTG	
13	F	GAAAGAGAGAGAGGGAACGA	<i>WOR1</i> deletion
14	R	CACGGCGCGCCTAGCAGCGGGATGTTTGAGATGT CAGTGTAC	
15	F	GTCAGCGCCGCATCCCTGCTATATGTGGGTCTG TGTGTG	
16	R	CGACGCGTTACCCATACGATGTTC	
19	F	CGGGATCCGGATCCTCTAGATTAGTAG	<i>HIS1</i> amplification
20	R	GTAGCGGCCGCATCGATAAGCTTTGGGTAC	
21	F	GCT CTAGA ATGTCTAATTCAAGTATAGTCC	<i>WOR1</i> FLAG addition
22	R	GG GTACCT ATTACTTGTTCATCGTCATCCTTGTA ATCGATGTCATGATCTTTATAATCACCGTCATGGT CTTTGTAGTCAGTACCGGTGTAATACGACC	
23	F	CGTAAACTATTTTTAATTTGAAAAATCAAAGAGGCA TACGGTTTTAGAGCTAGAAATAGC	<i>TUP1</i> sgRNA generation
24	R	GCTATTTCTAGCTCTAAAACCGTATGCCTCTTTGA TTTTTCAAATTAATAATAGTTTACG	
25	F	GTGTATGACTTGGAGTTGGCCATAGAAAAATCAA AGAGGCATACGAATAAGGATCCGG	<i>TUP1</i> repair template
26	R	CTCTAGTGTCCAACCTCGTTCTTTAACCTCAATATCT CTTCCGGATCCTTATTCGTATGC	
27	F	CGAGTACATTTTGTCCGGC	<i>TUP1</i> qPCR
28	R	GTTTAGGGATACAGCGACTG	
29	F	GCCAGATGGAAATCAGCAAC	<i>WOR2</i> qPCR
30	R	GGTTGAAGATCCAGAGATCG	
31	F	CCTCCACCACCTTTAATGAC	<i>WOR3</i> qPCR

32	R	CCATACCATTGTTTGGTTGCTG	
33	F	TGGAAGCTGCTGGTATTGAC	<i>ACT1</i> qPCR
34	R	TTCAGCAATACCTGGGAACA	
35	F	AAATAAACCATCGTCATCGGC	<i>WOR1</i> CDS qPCR
36	R	AGGACCATTACCTAGACTCG	
37	F	CCCTGACATCACCTTTGTTG	<i>WOR1</i> 5' UTR qPCR
38	R	TTCCCTTCCGTTAAATAACCC	
39	F	CAAGCAAACAAACGACCAAA	<i>EFG1</i> qPCR
40	R	CAAGGGACACAAAGGGGTTA	
41	F	CTCGGTGGCAGCTCTAGTTC	<i>OP4</i> qPCR
42	R	TCGGCAGCTTCCATAATTC	
43	F	ATCGAATCCAAATTAACCTCCAG	<i>WH11</i> qPCR
44	R	GAAGTAGCTTTACCAGCAGC	
45	F	GGGCGAAGGAGAGAGAATTA	<i>pWOR1</i> -10 kb
46	R	CCTGCAACATCACGTTAGAAA	
47	F	AGTGAGCAGCCAATTCAGC	<i>pWOR1</i> -7.4kb
48	R	GCAAAGGTGGTGAGCTAGAAG	
49	F	TGGTTGTTGTTGTTTATTCAGAATTT	<i>pWOR1</i> -6kb
50	R	TTTCCCACCCGTCTTTCATA	
51	F	CAAATTGAGACTTTAGATATGGT	<i>pWOR1</i> -4kb
52	R	TATGCCGTTTTCAATCAAATTATT	
53	F	ATGTCTATCTATATTCAAGTATC	<i>pWOR1</i> -2.8 kb
54	R	TTTGAGATGAGATGACGTTATAT	
55	F	TGTGTATAGTCTGTGCAGACT	<i>pWOR1</i> -2.4 kb
56	R	TTTTTTGTTTTTAAATAAAGTCAAAG	
57	F	CAGATAGACATTTCAATTAAGCA	<i>pWOR1</i> -2 kb
58	R	AGTTGTGTTAAATAATTCAATATTTA	
59	F	CAAAGTTTGGAGGGTACTAAACA	<i>pWOR1</i> 0 kb
60	R	GGGTGGTTCACGTTTGT	
61	F	TGATGGTAGAGGTAACCTTG	<i>ADE2</i>
62	R	TTTGGCTAATATAGATGCCT	

Table 2.4: Primers used in Chapter 4

Number	Direction	Sequence (5'-3')	Purpose
62	F	CGTAAACTATTTTTAATTTGTCAGTTTGTCCAGTAG GTGGGTTTTTAGAGCTAGAAATAGC	<i>grx1</i> deletion
63	R	GCTATTTCTAGCTCTAAAACCCACCTACTGCACAAA CTGACAAATTAATAATAGTTT	
64	F	GTGAGAGTTAATAGTGTGTTTCGATTTCTTTCTCAGT TTGTGCAGTAGGTGGTTAGGA	
65	R	TGCCTGGGGATTCAATTTGTGGTATCAACCAGGAT CCTAACCACCTACTGCACAAAC	
66	F	CGTAAACTATTTTTAATTTGGTTTTACAAGGTCCTG CCCAGTTTTAGAGCTAGAATAGC	<i>grx3</i> deletion
67	R	GCTATTTCTAGCTCTAAAACCTGGGCAGGACCTTGT AAAACCAAATTAATAATAGTTTA	
68	F	ATCCAAGTTGATTCGACTTTATTTCCATACAGGATC CTAATGGGCAGGACCTTGTA	
69	R	ATCAGCTAAGGTTTTGAATACTTGATTCATGGTTTT ACAAGGTCCTGCCCATTAGGA	
70	F	CCCAACTTTTTGCAGTGGTTTAACTAATAATCAACT ACAATGCCTACCTCACCTACA	<i>pMAL2-SFU1- MYC</i> generation
71	R	CACCGTTCAAGTCTTCCTCGGAGATTAGCTTTTGTT CTCCATTTAACAACCTCCCAAT	
72	F	TTGCAGTGGTTTAACTAATAATCAACTACATCTAGA ATGAAGTTTGAAAAAGGTAAAGTG	<i>pMAL2-SEF1- GFP</i> generation
73	R	ACCAGTGAATAATTCTTCACCTTTAGACATACGCGT TTTCTCTTGCATCATATTAACATC	
74	F	GAATTGTAATACGACTCACTATAGGGCGAATTGGG TACCAATGGTGGGTGTGTTTAT	<i>pGRX3-GRX3- FLAG</i> generation
75	R	AGCTTCTAAAGCATGTTCTGAAG	
76	F	TTGAAGATGATGAGAAATTCTTCGAACATGCTTTAG AAGCTGACTACAAAGACCATG	
77	R	CTCTAGTTTTGACGGGATCCCCGGGCTGCAGTCA CTTGTGCATCGTCATCCTTGTAAT	
78	F	ACGGGAATTACGTTCTCAACAG	<i>pSEF1</i> qPCR
79	R	GTTAAGGGAGGAGTCGATTGG	
80	F	GCACCGCTCTCTCATTACC	<i>SFU1</i> qPCR
81	R	GTGGTTGTGATTGCGTCGGT	
82	F	TCGATCTGCCACCGAATTGT	<i>SEF1</i> qPCR
83	R	TCTCCACCACGATGTAGACCT	
84	F	GGCTGTACCGGTAATCCAGGAA	<i>HAP43</i> qPCR
85	R	ACCGACGAAGAGTGGGATGG	
86	F	GGTAGCTGGTCCTGCCGCTA	<i>CCP1</i> qPCR
87	R	ACCTGATGTATGCCATGCCAATCT	
88	F	TGATGGTAGAGGTAACCTTG	<i>ADE2</i> qPCR
89	R	TTTGGCTAATATAGATGCCT	
90	F	TGGAAGCTGCTGGTATTGAC	<i>ACT1</i> qPCR
91	R	TTCAGCAATACCTGGGAACA	

CHAPTER 3

Wor1 establishes opaque cell fate through inhibition of the general co-repressor Tup1 in *Candida albicans*

INTRODUCTION

Candida albicans is a common opportunistic fungal pathogen of humans. Found in the mouth, gastrointestinal tract, vagina and skin, *C. albicans* is harmless to the healthy, but in immunocompromised individuals *C. albicans* can cause serious infection, with a mortality rate of up to 40% in disseminated systemic infections [86]. *C. albicans* is able to transition between several phenotypic forms. This ability allows *C. albicans* to easily adapt to and inhabit various diverse host niches [86]. These different phenotypic states display not only different morphological features such as cell shape and size, but also altered metabolism and gene expression patterns. One such transition is the switch between the white and opaque states [87], which is regulated by many different stimuli such as N-acetylglucosamine [10, 88], high CO₂ [11], hypoxia [12], temperature [3, 13], and genotoxic stress [14-16]. The opaque phase is the mating competent form of *C. albicans* and is inhibited by the a1- α 2 repressor complex [17, 18]. In addition to genes related to mating, white and opaque cells show differential regulation of hundreds of genes involved in metabolism, adhesion, cell surface composition, stress response, signaling, and virulence [4, 5]. Opaque cells generally show reduced virulence compared to white cells in a systemic setting, however, opaque cells are excellent

colonizers of the skin and show increased fitness in cutaneous infection models [6, 7]. Additionally, opaque cells are less efficiently phagocytosed than white cells, implicating phase switching in host immune evasion [8, 9]. More recently, white-opaque switching has been observed in *MTLa/a* strains by passage through the mammalian gut [20] and in certain *MTLa/a* clinical isolates [19]. These *a/a* opaque cells are somewhat distinct from their mating-competent *MTL* homozygous counterparts, and show enhanced commensalism compared to white cells in the gut [20].

On the molecular level, the opaque state is induced through expression of the master regulator *Wor1*, which regulates its own expression and forms a stable autoregulatory feedback loop to induce and maintain the opaque state [21-23]. *Wor1* is part of an extensive underlying network of transcriptional regulators governing white-opaque switching [28, 30, 31, 89]. While some regulators show differential phase expression, all identified white-opaque regulators show opaque-enriched binding at each other's promoter regions [28]. This is most evident at the 8kb-long *WOR1* promoter, where both positive and negative regulators of the opaque phase share enhanced binding at the same regions along the active *WOR1* promoter. In addition, white-opaque switching is also regulated at the levels of promoter chromatin and protein translation [15, 16, 24-27]. *Wor1* belongs to a class of conserved fungal transcription factors, many of which have been identified as master regulators of morphological development and virulence in pathogenic fungi, such as *Ryp1* in the human pathogen *Histoplasma capsulatum* [35] and *Sge1* in the plant pathogen *Fusarium oxysporum* [34]. This family of proteins contains an uncharacterized C-terminal domain and two unique, highly conserved N-terminal domains termed *WOPRa* and *WOPRb*, both of which are

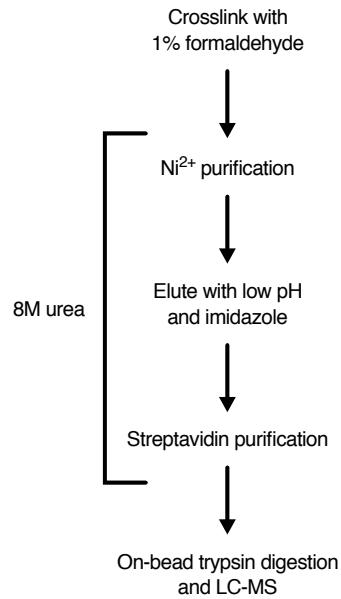
essential for the sequence-specific DNA binding properties of this class of protein [29, 33, 36]. Point mutation of these domains leads to disruption of Wor1 DNA binding and its ability to induce the opaque phase [29, 36]. How Wor1 functions to induce and sustain the opaque state is not known.

Transcriptional repression is frequently used to control gene expression in cell fate determination. The general repressor Tup1 is a member of the highly conserved Gro/TLE family of proteins that are present in many species across several kingdoms [90, 91]. It does not bind DNA directly but instead is recruited to different genomic regions through interactions with sequence-specific transcriptional regulators to repress many diverse sets of genes [92, 93]. Tup1 inhibits gene expression through several mechanisms, including recruitment of histone deacetylases, chromatin remodeling, and modulation of Mediator function [94, 95]. In this study we use a global mass spectrometry-based approach to identify proteins associated with Wor1 in order to understand how Wor1 functions as the master regulator of the opaque phase. We identify the general co-repressor Tup1 as a Wor1-interacting protein and the key repressor of the opaque state. We further demonstrate that external cues, such as temperature and carbon source, regulate Wor1 and Tup1 at the *WOR1* promoter to control cell fate.

RESULTS

Tandem affinity purification of proteins cross-linked to Wor1 identifies Tup1 complexes

In an effort to identify key regulators and mechanisms governing white-opaque switching, we employed a biochemical approach to better understand how Wor1 functions as the master regulator of the opaque phase. We set out to identify Wor1 interacting proteins by utilizing cross-linked tandem affinity purification in denaturing conditions coupled with mass spectrometry using a His-Biotin-His (HBH) tag cloned onto the C-terminal end of Wor1 (Figure 3.1A). The HBH tag, originally designed for use in *S. cerevisiae* [71], was codon optimized for efficient expression in *C. albicans*, a member of the CTG clade [96]. The benefit of using this tandem affinity purification method is two-fold. Firstly, cross-linking allows us to capture weak and transient interactions that may otherwise be missed; secondly, the ability to perform both purification steps under denaturing conditions helps to prevent protein degradation and loss of post-translational modifications. *C. albicans* cells carrying a *MAL2* promoter driven copy of the C-terminally HBH-tagged Wor1 were grown in maltose-containing media overnight then cross-linked with 1% formaldehyde, washed, lysed in 8M urea buffer, and the resulting clarified protein extracts were incubated with Ni^{2+} agarose beads. The bound proteins were then eluted and incubated with streptavidin-conjugated agarose beads. Samples were treated to an on-bead trypsin digestion and the resulting peptides were analyzed by LC-MS (Figure 3.1A). As a negative control, the same procedure was performed without cross-linking to determine which proteins are bound without crosslinking, such as those covalently linked to Wor1 or non-specifically interact with beads. Another negative control was performed with cross-linking, but using a strain without HBH-tagged Wor1 to determine which proteins nonspecifically interact with the Ni^{2+} - and streptavidin-conjugated agarose beads. Proteins found associated

A**B**

ORF	Name	Function
orf19.6109	<i>TUP1</i> [§]	Transcriptional co-repressor
orf19.670	<i>SMT3</i> [*]	SUMO, small ubiquitin-like protein
orf19.395	<i>ENO1</i>	Enolase
orf19.6090	<i>NSR1</i>	Nucleolar protein
orf19.6798	<i>SSN6</i> [§]	Homolog of ScCyc8
orf19.1065	<i>SSA2</i>	HSP70 family chaperone
orf19.2551	<i>MET6</i>	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase
orf19.6734	<i>TCC1</i> [§]	<i>Candida</i> -specific Ssn6 ortholog
orf19.4623.3	<i>NHP6A</i>	Non-histone chromatin component
orf19.3942.1	<i>RPL43A</i>	Ribosomal protein
orf19.798	<i>TAF14</i>	DNA-binding transcription factor
orf19.542	<i>HXK2</i>	Hexokinase II
orf19.5137.1	<i>HHO1</i>	Histone H1
orf19.3575	<i>CDC19</i>	Pyruvate kinase at yeast cell surface
orf19.5112	<i>TKL1</i>	Transketolase
orf19.7308	<i>TUB1</i>	Alpha-tubulin
orf19.3324	<i>TIF</i>	Translation initiation factor
orf19.467	<i>WOR3</i>	White-opaque regulator 3
orf19.7676	<i>XYL2</i>	D-xylulose reductase
orf19.2994	<i>RPL13</i>	Ribosomal subunit
orf19.4959		Unknown function
orf19.4632	<i>RPL20B</i>	Ribosomal protein L20
orf19.1354	<i>UCF1</i>	Unknown function
orf19.7350	<i>RCT1</i>	Unknown function
orf19.657	<i>SAM2</i>	S-adenosylmethionine synthetase
orf19.3037	<i>PAB1</i>	Poly(A)-binding protein
orf19.2364	<i>MIS11</i>	Mitochondrial C1-tetrahydrofolate synthase precursor

Figure 3.1. Identification of Wor1 interacting proteins by tandem affinity HBH purification coupled with mass spectrometry. (A) The workflow for an HBH purification in denaturing conditions utilizing formaldehyde cross-linking. **(B)** A list of Wor1 interacting proteins compiled from five HBH purifications. All proteins listed were observed only in Wor1-HBH (HLY4532) purifications with crosslinking in three or more of the purifications performed. Proteins marked by § are known components of the Tup1 co-repressor complex. Proteins marked with an asterisk were also observed in Wor1-HBH purifications without cross-linking. A complete list of identified proteins in each Wor1-HBH purification is provided in Table 3.1. Proteins are ranked in descending order first by number of times observed in independent purifications and then by average normalized iBAQ (intensity-based absolute quantification) value. Predicted functions are derived from the *Candida* Genome Database.

Table 3.1: Wor1 interacting proteins identified by mass spectrometry

Wor1-HBH purification in cross-linking conditions

	Purification no.	1	2	3	4	5	Times seen	Average iBAQ
	Sonication (Y/N)	N	N	N	N	Y		
Protein ID	Accession							
WOR1*	orf19.4884	4.49E-01	6.25E-01	2.95E-01	1.52E-01	7.56E-02	5	3.19E-01
TUP1 [§]	orf19.6109	5.34E-03	5.51E-03	9.43E-02	3.15E-02	4.41E-02	5	3.61E-02
SMT3*	orf19.670	1.42E-02	2.51E-02	5.35E-03	4.18E-02	1.32E-02	5	1.99E-02
ENO1	orf19.395	3.99E-03	7.95E-03	9.72E-03	1.35E-02	1.87E-02	5	1.08E-02
NSR1	orf19.6090	2.83E-04	4.74E-04	6.19E-03	7.89E-03	1.10E-02	5	5.16E-03
SSN6 [§]	orf19.6798	2.50E-04	2.72E-04	2.41E-03	5.11E-03	2.83E-03	5	2.17E-03
SSA2	orf19.1065	3.64E-04	5.39E-04	4.66E-03	2.42E-03	1.60E-03	5	1.92E-03
MET6	orf19.2551	6.61E-04	9.29E-04	1.17E-03	3.29E-03	2.48E-03	5	1.70E-03
TCC1 [§]	orf19.6734	2.30E-04	2.62E-04	3.42E-03	2.36E-03	1.64E-03	5	1.58E-03
NHP6A	orf19.4623.3	3.89E-03	5.39E-03		2.40E-02	3.40E-02	4	1.68E-02
RPL43A	orf19.3942.1		1.78E-03	4.90E-03	2.85E-02	2.19E-02	4	1.43E-02
TAF14	orf19.798	3.27E-04		2.48E-02	1.95E-03	5.09E-03	4	8.04E-03
HXK2	orf19.542	2.31E-04	5.84E-04		2.28E-03	5.17E-03	4	2.06E-03
HHO1	orf19.5137.1	2.02E-04	3.17E-04		2.94E-03	2.37E-03	4	1.46E-03
CDC19	orf19.3575	1.60E-04	1.16E-03		5.37E-04	1.59E-03	4	8.64E-04
TKL1	orf19.5112	2.20E-04	3.21E-04	2.24E-03		2.71E-04	4	7.63E-04
TUB1	orf19.7308	1.02E-04	5.83E-05		1.11E-03	1.54E-03	4	7.02E-04
TIF	orf19.3324	2.07E-04	2.50E-04	9.04E-04		4.66E-04	4	4.57E-04
WOR3	orf19.467	3.86E-05			4.70E-03	5.56E-03	3	3.43E-03
XYL2	orf19.7676		6.67E-05		3.58E-03	3.18E-03	3	2.27E-03
RPL13	orf19.2994	1.67E-04	1.05E-04	2.85E-03			3	1.04E-03
orf19.4959	orf19.4959	9.78E-04	3.25E-05		1.69E-03		3	8.99E-04
RPL20B	orf19.4632	6.90E-04	1.02E-04	1.84E-03			3	8.78E-04
UCF1	orf19.1354	2.37E-04	1.22E-04			1.65E-03	3	6.71E-04
orf19.7350	orf19.7350	1.72E-04	1.91E-04	1.46E-03			3	6.06E-04
SAM2	orf19.657		6.32E-05	1.47E-03		2.59E-04	3	5.98E-04
orf19.3037	orf19.3037	5.36E-05			6.47E-04	1.49E-04	3	2.83E-04
MIS11	orf19.2364	1.18E-04	7.68E-05			4.56E-04	3	2.17E-04
NRG1	orf19.7150				7.42E-03	9.03E-03	2	8.23E-03
RPS12	orf19.6785		2.26E-04	7.83E-03			2	4.03E-03
PTC2	orf19.2538				6.92E-04	6.16E-03	2	3.43E-03
WH11	orf19.3548.1	1.63E-03	1.62E-03				2	1.62E-03
RPL24A	orf19.3789	2.55E-04				2.40E-03	2	1.33E-03
orf19.173	orf19.173				7.99E-04	1.08E-03	2	9.39E-04
orf19.831	orf19.831	2.20E-05				1.30E-03	2	6.60E-04
ARD	orf19.6322				8.47E-04	3.62E-04	2	6.05E-04
GPM1	orf19.903		9.50E-05			1.00E-03	2	5.48E-04
PGI1	orf19.3888		5.37E-05	9.69E-04			2	5.11E-04

TPI1	orf19.6745		2.54E-04			4.79E-04	2	3.67E-04
TUB2	orf19.6034	1.05E-04				5.25E-04	2	3.15E-04
orf19.4216	orf19.4216	9.00E-05	4.08E-04				2	2.49E-04
EFG1	orf19.610	1.98E-04	1.73E-04				2	1.86E-04
PMM1	orf19.2937		1.58E-04			1.77E-04	2	1.67E-04
PHO4	orf19.1253	3.65E-05				2.06E-04	2	1.21E-04
RNR21	orf19.5801	1.18E-04	9.54E-05				2	1.07E-04
HSP90	orf19.6515	9.16E-05	5.04E-05				2	7.10E-05
STP2	orf19.4961	2.81E-05				1.13E-04	2	7.03E-05
LYS22	orf19.4506	5.14E-05	6.43E-05				2	5.79E-05
SPT5	orf19.1453	1.23E-05	1.50E-05				2	1.37E-05
orf19.2330	orf19.2330			1.14E-01			1	1.14E-01
PRE2	orf19.2233			1.92E-02			1	1.92E-02
SKP1	orf19.4427		8.37E-03				1	8.37E-03
orf19.1536	orf19.1536			5.06E-03			1	5.06E-03
orf19.3545	orf19.3545			3.18E-03			1	3.18E-03
orf19.2755	orf19.2755			2.59E-03			1	2.59E-03
orf19.2740	orf19.2740			1.86E-03			1	1.86E-03
orf19.3606	orf19.3606					1.75E-03	1	1.75E-03
orf19.6502	orf19.6502			1.56E-03			1	1.56E-03
orf19.3233	orf19.3233					1.44E-03	1	1.44E-03
orf19.5614	orf19.5614			9.97E-04			1	9.97E-04
orf19.4399	orf19.4399		9.05E-04				1	9.05E-04
orf19.2341	orf19.2341	8.74E-04					1	8.74E-04
RPS17B	orf19.2329.1					7.75E-04	1	7.75E-04
RPN6	orf19.1299			7.23E-04			1	7.23E-04
RPS4A	orf19.5341	7.08E-04					1	7.08E-04
orf19.1082.1	orf19.1082.1					5.85E-04	1	5.85E-04
GZF3	orf19.2842					5.55E-04	1	5.55E-04
RAV2	orf19.5561		5.24E-04				1	5.24E-04
orf19.1625	orf19.1625	4.21E-04					1	4.21E-04
RPS21	orf19.3334					4.16E-04	1	4.16E-04
orf19.5514	orf19.5514					3.52E-04	1	3.52E-04
CBF1	orf19.2876					3.39E-04	1	3.39E-04
YHB1	orf19.3707					3.16E-04	1	3.16E-04
orf19.2097	orf19.2097			2.98E-04			1	2.98E-04
ASN1	orf19.198	2.83E-04					1	2.83E-04
HMO1	orf19.6645		2.76E-04				1	2.76E-04
orf19.4088	orf19.4088	2.60E-04					1	2.60E-04
LSC2	orf19.1860					2.39E-04	1	2.39E-04
ZCF31	orf19.5924					2.22E-04	1	2.22E-04
NOP1	orf19.3138	1.94E-04					1	1.94E-04
RPL35	orf19.5964.2	1.80E-04					1	1.80E-04
CYT1	orf19.3527		1.74E-04				1	1.74E-04
WHI3	orf19.6494	1.53E-04					1	1.53E-04
PCK1	orf19.7514					1.52E-04	1	1.52E-04
MAL2	orf19.7668	1.38E-04					1	1.38E-04
GLY1	orf19.986	1.28E-04					1	1.28E-04
SNT1	orf19.5241					9.63E-05	1	9.63E-05

orf19.7263	orf19.7263	9.11E-05					1	9.11E-05
orf19.1897	orf19.1897		6.62E-05				1	6.62E-05
GPH1	orf19.7021	6.48E-05					1	6.48E-05
orf19.3815	orf19.3815	6.13E-05					1	6.13E-05
orf19.5239	orf19.5239	4.57E-05					1	4.57E-05
GLN1	orf19.646	4.32E-05					1	4.32E-05
NOP5	orf19.1199	4.10E-05					1	4.10E-05
IPP1	orf19.3590	3.92E-05					1	3.92E-05
KTR4	orf19.4475	3.47E-05					1	3.47E-05
GCV2	orf19.385	3.06E-05					1	3.06E-05
PMC1	orf19.1727	2.68E-05					1	2.68E-05
orf19.5871	orf19.5871	2.67E-05					1	2.67E-05
orf19.5408	orf19.5408		2.58E-05				1	2.58E-05
orf19.4347	orf19.4347	1.58E-05					1	1.58E-05
orf19.1777	orf19.1777	6.57E-06					1	6.57E-06

*Observed in Wor1-HBH purifications without cross-linking.

§ Known components of the Tup1 co-repressor complex.

Table 3.1. Wor1 interacting proteins identified by mass spectrometry. Proteins listed are identified only in Wor1-HBH purifications performed with cross-linking, but not in non- cross-linked or untagged purifications. Proteins are ranked in descending order first based off number of times identified in Wor1-HBH cross-linked purifications and then by decreasing average normalized iBAQ (intensity-based absolute quantification) value derived via the Max-Quant suite. Proteins marked by § are known components of the Tup1 co-repressor complex. Proteins marked with an asterisk were also observed in Wor1-HBH purifications without cross-linking.

with Wor1-HBH in 3 out of 5 biological replicates were ranked by intensity-based absolute quantification (iBAQ) and are shown in Figure 3.1B [83]. A complete list of identified proteins is provided in Table 3.1.

The general transcriptional co-repressor Tup1 consistently ranked as the top Wor1-associated protein (Figure 3.1B). In *C. albicans*, the Tup1 co-repressor complex can exist in two forms, either with Ssn6 or with Tcc1, which are both homologs of *S. cerevisiae* Cyc8 [97-99]. Both Ssn6 and Tcc1 were top ranked in all 5 purifications (Figure 3.1B). Also observed were chromatin-associated proteins Non-Histone Protein 6A (Nhp6a), which is commonly associated with actively transcribed genes [100], and Taf14, which is a subunit of several complexes including the INO80, SWI/SNF, and NuA3 complexes (Figure 3.1B, Table 3.1) [101]. The white-opaque regulator Wor3 was found in 3 out of 5 experiments, and although Wor3 is not known to interact with Wor1, both bind to same promoter regions of *WOR1* and other phase regulators [89]. Unlike other interacting proteins, Smt3 was identified as Wor1-associated in denaturing conditions without cross-linking (Figure 3.1B). This demonstrates that Wor1 is SUMOylated, which is consistent with a recent publication [102].

Tup1 is bound to the *WOR1* promoter preferentially in opaque cells

To determine if Tup1 interacts with Wor1, we carried out immunoprecipitation experiments in native conditions without cross-linking in opaque cells. Despite many attempts, we were unable to observe a Wor1-Tup1 interaction in native conditions by IP with either Tup1-HA or Wor1-FLAG. Failure to observe binding in native conditions could mean that the interaction is too weak to capture by native IP or that the two

proteins are brought to interact through association at the chromatin. To test the latter possibility, we performed ChIP with a C-terminal 3xHA tagged Tup1 in white and opaque cells, and examined Tup1 binding at several regions along the *WOR1* promoter where Wor1 and other key white-opaque transcriptional regulators have been shown to bind [28]. We found that Tup1 binds to the *WOR1* promoter at 7.4kb, 6kb, 4kb, and 2.4kb upstream of the *WOR1* TSS preferentially in opaque cells despite having the same levels of Tup1 protein in both phases (Figure 3.2A, Figure 3.3). This is consistent with the known binding profile of Wor1 [31]. Additionally, in white cells Tup1 is associated with the 2.4kb upstream region where Efg1 is known to bind (Figure 3.2B), and we also found Efg1 in 2 out of 5 Wor1-HBH purifications (Table 3.1) [28]. Our observation that Tup1 occupies the *WOR1* promoter preferentially in opaque phase as well as the inability to detect the Wor1-Tup1 interaction without cross-linking further supports the hypothesis that Tup1 interacts with Wor1 on chromatin.

Tup1 depletion in liquid culture leads to *WOR1* expression, and results in opaque formation when re-expressed

The identification of Tup1, Ssn6 and Tcc1 in all our cross-linked Wor1 purifications prompted us to examine the functions of Tup1 in opaque formation. Tup1 has previously been linked to phenotypic switching [103, 104]. Loss of Tup1 led to constitutively filamentous cell morphology, resulting in fuzzy and wrinkled colony morphologies. Loss of Tup1 was shown to induce the expression of downstream opaque gene *OP4*, although at a lower level than opaque cells. Additionally, expression of downstream white gene *WH11* remained high in the *tup1* mutants. Therefore, *tup1*

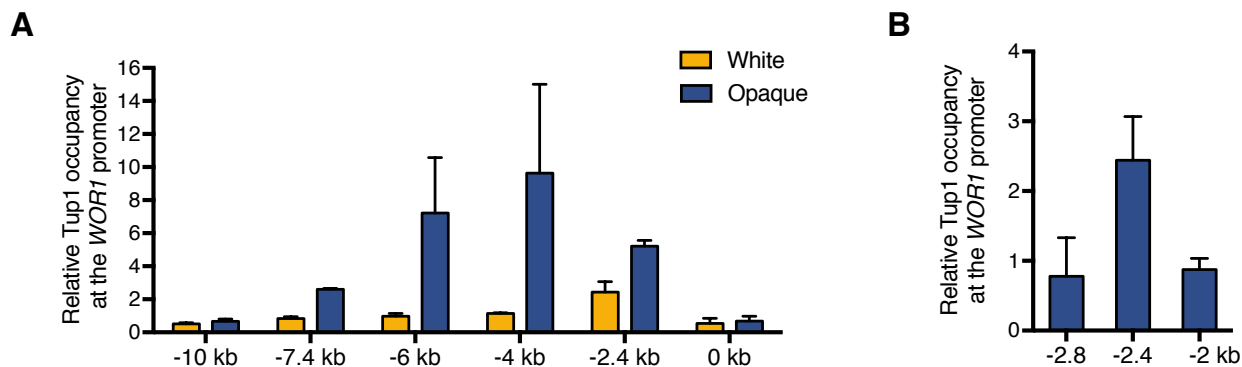


Figure 3.2. Tup1 binds along the *WOR1* promoter differentially in white and opaque phases. (A) ChIP of Tup1-HA in white and opaque cells. Overnight cultures of white and opaque cells of a wild-type strain (JYC5) and a strain carrying Tup1-HA (HLY4538) were diluted in SCD and grown to log phase at room temperature before formaldehyde. Enrichment is presented as a ratio of qPCR of the *WOR1* promoter IP (bound/input) over an *ADE2* control region IP (bound/input) of the tagged strain, further normalized to the control strain. Values are the average of three independent ChIP experiments with error bars representing the s.d. **(B)** Additional qPCR of Tup1 binding around -2.4kb upstream of the *WOR1* TSS in white cells from **(A)**.

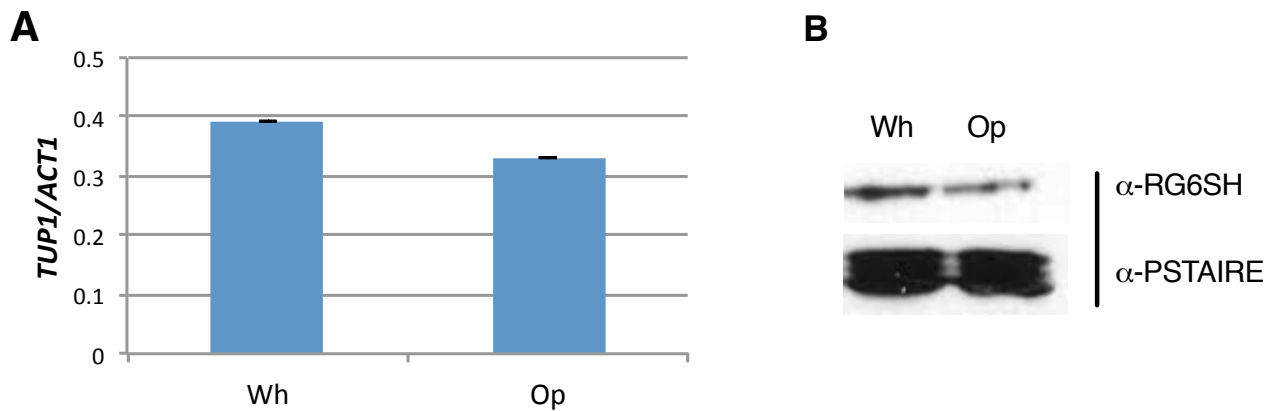


Figure 3.3. Tup1 levels are similar in white and opaque cells. **A.** *TUP1* expression level in white and opaque cells. Overnight cultures of WT white and opaque cells (JYC5) were inoculated and grown to log phase. *TUP1* expression level was measured by qPCR and normalized to *ACT1*. Expression values are the average of three independent qPCR experiments and error bars represent the s.d. **(B)** Tup1 protein level in white and opaque phases. Overnight cultures of a strain carrying *pTUP1-TUP1-HBH* (HLY4542) were inoculated into fresh YPD, grown to log phase. Protein level was assessed by Western blot as described.

mutants express both white and opaque genes [103, 104]. Further, Tup1 depletion then re-expression led to near complete conversion from white cells to opaque colonies [103].

Since these early studies were carried out before the discovery of Wor1 and other key transcriptional regulators of the white-opaque transition, we re-examined the role of Tup1 in white-opaque switching using a conditional *tup1* mutant, as in Zhao et al. [103]. We replaced *TUP1* with a *MET3* promoter-driven *TUP1* in the haploid strain GZY822 [75]. *TUP1* is expressed when grown in the absence of methionine, but repressed upon addition of methionine (Figure 3.4A Fig). Using this strain, we depleted Tup1 in white cells in liquid cultures, and then plated cells on methionine-containing plates to assay for opaque colonies under the condition of Tup1 re-expression. As had been previously shown in diploid cells [103], extended depletion in liquid Met⁺ medium followed by re-expression of Tup1 on Met⁻ plates led to near complete conversion of white cells to the opaque phase (Figure 3.5A). In support of the repressive function of Tup1 in opaque formation, ectopic overexpression of *TUP1* in opaque cells converted them back to the white phase on plates after 5-7 days of growth (Figure 3.5B). Therefore, Tup1 is a repressor of opaque formation.

Conversion to the opaque phase is slow, with only around 50% opaque formation by 24hr post-shutdown (Figure 3.5A). These data indicated that Tup1 protein is depleted slowly in the conditional mutant after promoter shutdown. In support of this, we find that Tup1 protein is still detectable at 24hr after promoter shutdown, suggesting that Tup1 protein is highly stable (Figure 3.4B Fig). Considering that *TUP1* expression levels are similar in white and opaque phases (Figure 3.3), and Tup1 protein is highly stable

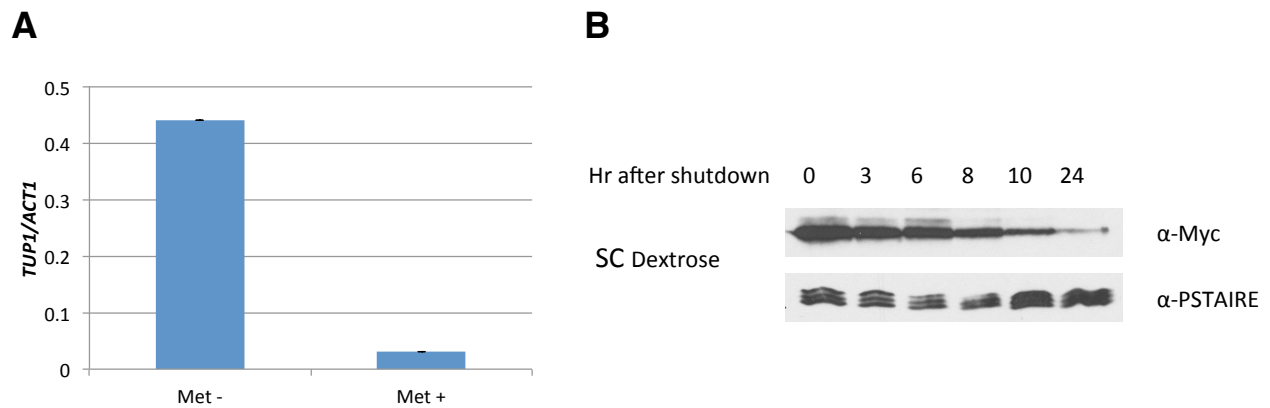


Figure 3.4. Verification of *pMET3-TUP1* construct and Tup1 protein stability. A. Shutdown of *TUP1* expression by methionine addition in the conditional mutant haploid strain carrying *pMET3-TUP1*. Overnight cultures of white *pMET3-TUP1* (HLY4533) cells grown in Met- SCD were diluted into fresh SCD with and without 5mM methionine. Cells were collected after 24hr and *TUP1* expression level was quantified by qPCR and normalized to *ACT1*. Expression values are the average of three independent qPCR experiments and error bars represent the s.d. **B.** Tup1 protein stability. White cells of a strain carrying *pMAL2-Tup1-Myc* (HLY4536) were grown in YPM overnight, washed with H₂O, and inoculated into YPD to shut down *pMAL2* promoter activity. Samples were taken at the indicated times and Tup1 protein level was assessed by Western blot, as described.

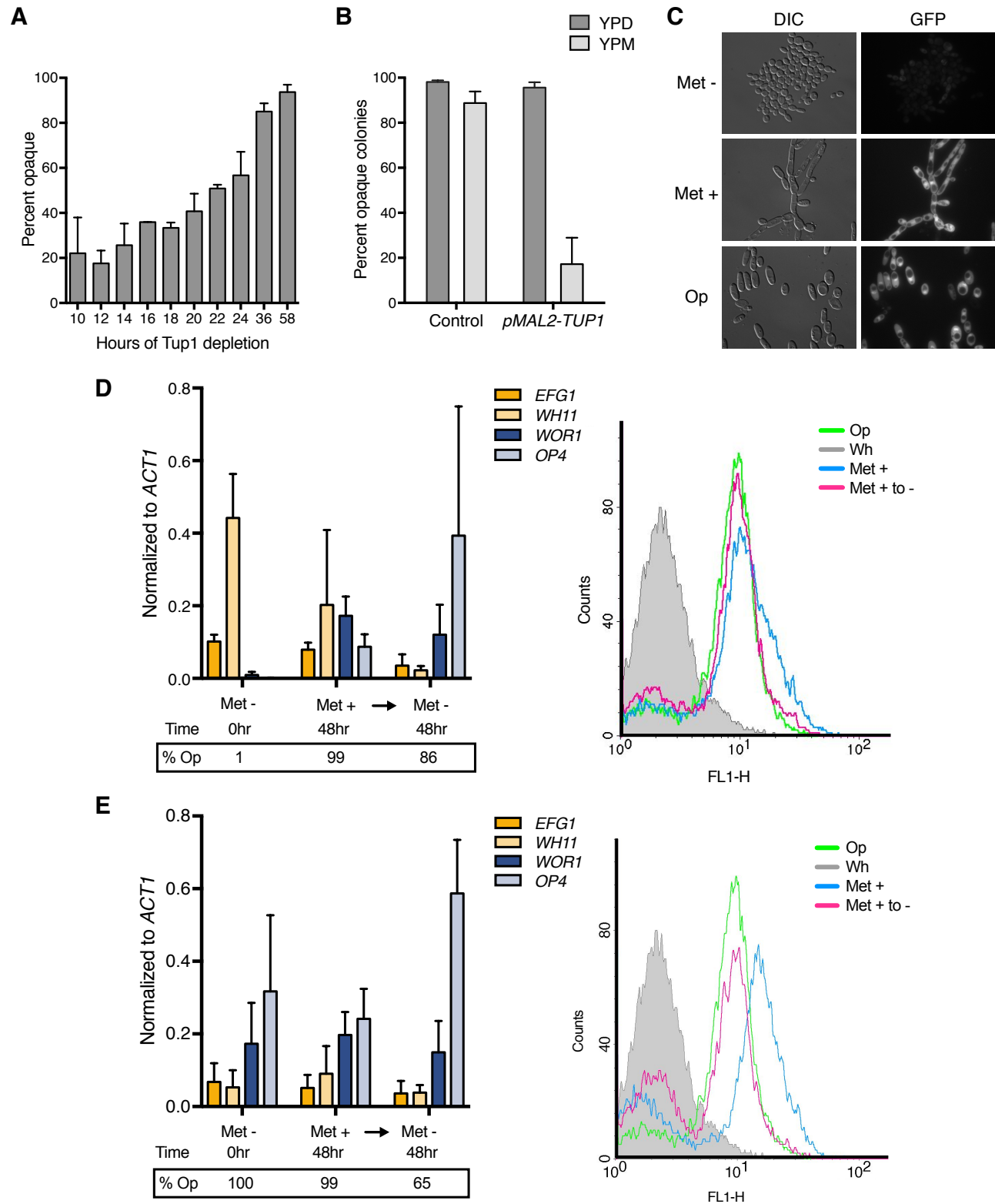


Figure 3.5. Tup1 is a repressor of the opaque state. (A) The effect of Tup1 depletion on opaque formation in liquid medium. White *pMET3-TUP1* (HLY4533) cells were depleted of Tup1 by growth in liquid SCD media containing methionine (Met +) for the indicated times and plated onto SCD media lacking methionine (Met -) plates to assay for opaque cells upon Tup1 re-expression. Plates were grown for 5-7 days at room temperature. **(B)** The effect of *TUP1* overexpression on opaque stability on solid medium. Opaque *pMAL2-TUP1* cells (HLY4537) were grown on SCM plates at room temperature for 5-7 days. Both whole and sectorized opaque colonies were counted as opaque. **(C)** *pWOR1-GFP* induction in Tup1 depleted cells. *pMET3-TUP1* cells carrying *WOR1* promoter driven GFP (HLY4535) were cultured in SCD with and without methionine for 48hr at room temperature and imaged for *pWOR1-GFP* expression. Opaque control cells were grown in the absence of methionine. **(D)** Gene expression levels and population dynamics of *pWOR1-GFP* expression in response to Tup1 depletion in starting white cells. White *pMET3-TUP1* cells carrying a *pWOR1-GFP* reporter (HLY4535) were grown in liquid Met + SCD medium for 48hr then washed and transferred to liquid Met - SCD medium and cultured for a further 48hr. Samples for qPCR analysis, plating, and flow cytometry were all taken at the same times, as indicated. The grey curve is starting white *pMET3-TUP1* cells at time 0hr; blue is 48hr in Met+ SCD; pink is 48hr in Met- SCD (Tup1 re-expression); green is an opaque control. **(E)** Gene expression levels and population dynamics of *pWOR1-GFP* expression in response to Tup1 depletion in starting opaque cells. Tup1 depletion procedure is the same as **(D)** except with starting opaque cells. Expression level in **(D)** and **(E)** was normalized to *ACT1* and the average expression level of three independent qPCR experiments are plotted with error bars representing the s.d.

(Figure 3.4B), we suggest that stochastic phase switching in *C. albicans* is not regulated through mechanisms that change Tup1 protein level.

To determine the effect of Tup1 depletion on the expression of *Wor1*, we introduced a *WOR1* promoter driven GFP reporter construct into the *pMET3-TUP1* conditional mutant strain such that GFP expression correlated with *WOR1* promoter induction. Fluorescent microscopy of white cells cultured in Met + medium at room temperature for 48hr showed cells exhibited robust *pWOR1-GFP* expression on par with opaque control cells (Figure 3.5C). Therefore, Tup1 depletion induces *WOR1* expression. Since loss of Tup1 leads to a constitutively filamentous phenotype, it is possible that formation of long chains of cells could confound the ability to determine single-cell switching events when cells are plated on solid media. However, we observed uniform GFP induction along Tup1-depleted cell chains and filaments, indicating that all cells in the Tup1-depleted filaments induced similar levels of *WOR1* expression. Therefore, even if an opaque colony did not result from a single cell but instead a filament cluster, all cells in the opaque colony-forming filament cluster would be opaque (Figure 3.5A).

We next used the conditional mutant reporter strain to assess switching dynamics in at the single cell level in liquid culture. White *pMET3-TUP1* cells carrying the *pWOR1-GFP* reporter were grown in liquid Met+ SCD medium for 48hr then washed and transferred to liquid Met- SCD medium and cultured for a further 48hr. Cells were collected for qPCR analysis, plating, and flow cytometry (Figure 3.5D). Based on the plating data, 99% of white cells had converted to opaque after 48hr of Tup1 depletion and showed expression of the opaque-specific genes *WOR1* and *OP4* (Figure 3.5D, left

panel). While *WOR1* and *OP4* were expressed in the absence of Tup1, the white-phase marker *WH11* was only partially repressed. However, re-expression of Tup1 completely repressed *WH11* expression while increasing *OP4* expression (Figure 3.5D, left panel). This data is consistent with published findings, with *tup1* cells showing *OP4* induction, although lower than in opaque cells, as well as simultaneous *WH11* expression that is only suppressed upon re-expression of Tup1 [103, 104]. The partial repression of *WH11* and induction of *OP4* in Tup1 depleted cells could be due to an averaging of cell populations in different cell phases. To determine if this was the case, we performed flow cytometry analysis and found that loss of Tup1 led to a single peak of *WOR1* transcription, indicating a single-population response to Tup1 depletion (Figure 3.5D, right panel), consistent with our earlier fluorescent microscopy findings (Figure 3.5C). These data demonstrate that Tup1 depletion causes white cells to lose repression of *WOR1* expression, but full expression of *OP4* and repression of *WH11* requires re-expression of Tup1.

We also depleted Tup1 from opaque cells using the same strain and experimental design. Opaque cells depleted of Tup1 remain opaque, both by gene expression in the absence of Tup1 in liquid media and by re-plating assay on methionine-lacking plates (Figure 3.5E). This unidirectional phase regulation is consistent with a previous report [103]. Therefore, Tup1 depletion in opaque cells does not show further impact on opaque-regulated gene expression. All together, we show that Tup1 depletion in liquid medium can induce *WOR1* expression, leading to an *en masse* white-to-opaque conversion upon Tup1 re-expression. Therefore, Tup1 is a key repressor of the opaque state.

Tup1 depletion bypasses the requirement for Wor1 in *WOR1* expression

Since we identified Tup1 as the key repressor of the opaque phase and as Wor1 is the master activator of the opaque phase, we were interested in functional relationship between the two regulators in opaque formation. To address if Tup1 depletion is sufficient to overcome the Wor1 requirement in opaque induction, we deleted the *WOR1* coding sequence in the conditional *pMET3-TUP1* strain. This strain is a conditional *tup1 wor1* double mutant when grown in methionine-containing medium. In addition, the *WOR1* 5' UTR remained intact in this strain, allowing us to assess transcription from the *WOR1* promoter using primers specific to the 5' UTR sequence. Depleting Tup1 in the parental *pMET3-TUP1* strain by growth in methionine-containing medium results in the expression of both opaque-specific regulators *WOR1*, *WOR2*, *WOR3*, as well as the opaque specific marker *OP4* (Figure 3.6A). In the conditional double mutant *pMET3-TUP1 wor1*, Tup1 depletion still induces the expression of the *WOR1* 5' UTR and other phase regulators *WOR2* and *WOR3*, like the parental *pMET3-TUP1* strain (Figure 3.6A). Transcription from the *WOR1* coding region is not detected in the conditional double mutant as expected because the CDS region is deleted from this strain. Thus, Tup1 depletion is sufficient to bypass the Wor1 requirement for the expression of *WOR1* and opaque phase regulators.

In order to further validate the results we observed in the haploid conditional double mutant strain, we constructed a *wor1 tup1* double mutant in a diploid background. Using the CRISPR-Cas9 system [76], we deleted *tup1* from a *MTLa/a wor1* mutant [21] and examined the expression of opaque genes in the resulting *wor1 tup1*

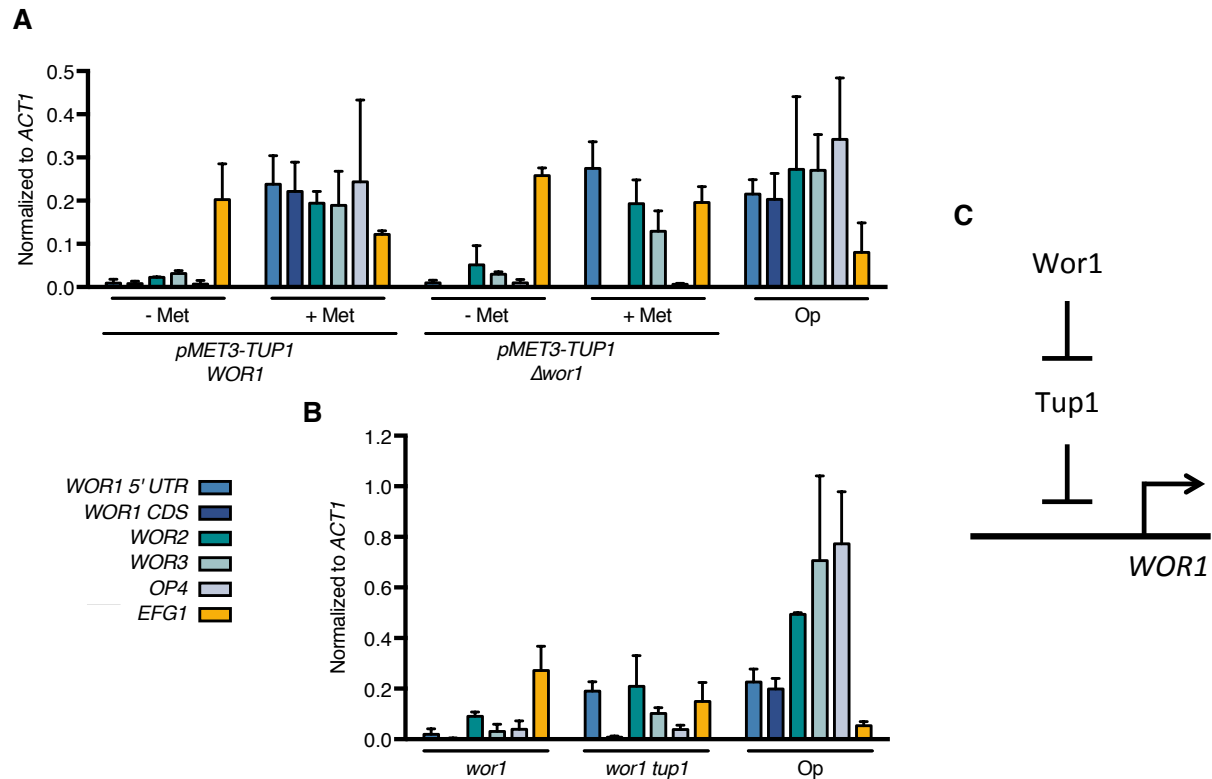


Figure 3.6. Tup1 depletion bypasses the requirement for Wor1 in the expression of *WOR1* and key opaque regulators. (A) Expression levels in the conditional Tup1 mutant and *wor1 pMET3-tup1* conditional double mutant haploid strains. *pMET3-TUP1* (HLY4533) and *pMET3-TUP1 wor1* (HLY4539) were grown at room temperature for 48hr in the presence or absence of methionine. **(B)** Expression levels in diploid *wor1* and *wor1 tup1* mutant strains. Overnight cultures of *MTLa/a wor1* (HLY3570), *wor1 tup1* (HLY4540), and a control strain (JYC1) were inoculated into fresh SCD and grown to log phase. Expression levels of the indicated genes in **(A)** and **(B)** were measured by qPCR and normalized to *ACT1*. *WOR1* expression was measured by qPCR using primers specific to either the *WOR1* 5' UTR or the *WOR1* coding region (CDS). Average expression level of three independent qPCR experiments was plotted with error bars representing the s.d. **(C)** Genetic model of Wor1-Tup1 regulation of *WOR1* expression.

double mutant strain (Figure 3.6B). Most notably, as in the haploid conditional double mutant, we see induction of *WOR1* 5'UTR expression in the *wor1 tup1* double mutant, but not in the *wor1* single mutant. Additional opaque-specific regulators *WOR2* and *WOR3* are also upregulated in the *wor1 tup1* mutant, although to varying degrees. However, loss of Tup1 in both the haploid and diploid double mutant strains does not induce *OP4* expression, unlike in the *pMET3-TUP1* single deletion strain. This implies that Tup1 does not directly regulate the expression of downstream opaque phase markers, which instead are under the control of Wor1.

This is the first report of a bypass of the master regulator Wor1 in the expression of opaque-specific regulators. This strongly suggests that a major function of Wor1 in opaque formation is to overcome the repressive activity of Tup1 and that Tup1 functions downstream of Wor1 (Figure 3.6C). This functional relationship predicts that some of the signals known to affect white-opaque switching that are believed to act on Wor1 may work through regulating Tup1.

Wor1 dissociates from the *WOR1* promoter upon temperature shift to 37°C

One phenomenon regarding phase switching is the complete and synchronous conversion of opaque cells back to the white phase when cultured at high temperatures[105]. Once cells are shifted to 37°C, *WOR1* transcription begins to decrease around 1.5hr, but commitment to the white phase doesn't occur until around 4-5 hours after temperature shift [106], yet Wor1 protein level is still high at this point (Figure 3.7A). How *WOR1* transcription decreases when Wor1 protein is still present is

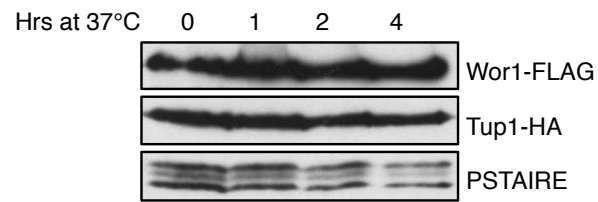
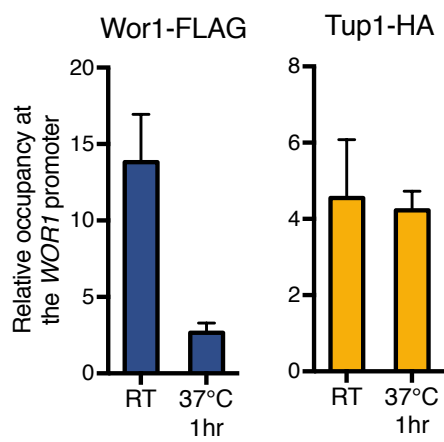
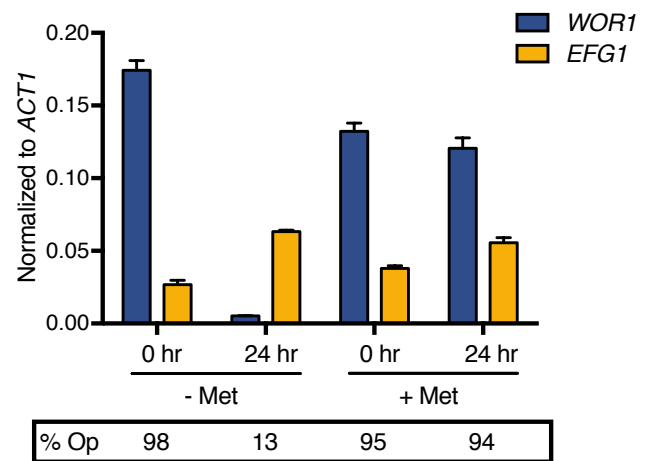
A**B****C**

Figure 3.7. Tup1 depletion stabilizes the opaque state even at 37°C. (A) Time course of Wor1 and Tup1 protein levels in opaque cells after shift from room temperature to 37°C. Overnight cultures of opaque cells of a strain carrying both Wor1-FLAG and Tup1-HA (HLY4541) were inoculated, grown to mid-log phase, then shifted to 37°C and grown for the indicated times. Protein level was assessed by Western blot as described. (B) ChIP of Wor1-FLAG and Tup1-HA at the *WOR1* promoter in opaque cells shifted to room temperature or 37°C. Overnight cultures of opaque cells of a strain carrying both Wor1-FLAG and Tup1-HA (HLY4541) and an untagged control strain (JYC1) were diluted in SCD and grown to log phase at room temperature. Cultures were divided and incubated at either room temperature or 37°C for one hour, formaldehyde cross-linked, and harvested for ChIP. Enrichment is presented as a ratio of the -4kb region of the *WOR1* promoter IP (bound/input) over an *ADE2* control region IP (bound/input) of the tagged strain, further normalized to the control strain. Values are the average of three independent ChIP experiments with error bars representing the s.d. (C) Tup1 depletion in opaque cells at room temperature and 37°C. Opaque *pMET3-TUP1* cells were grown in SCD with or without methionine at either room temperature or 37°C for 24hr. Expression levels of the indicated genes were measured by qPCR and normalized to *ACT1*. Average expression level of three independent qPCR experiments are plotted with error bars representing the s.d. Samples were also taken at the indicated times, washed three times with H₂O, and plated onto SCD Met- plates to assess phase switching.

unknown. ChIP of Tup1-HA and Wor1-FLAG in opaque cells at room temperature and at 37°C showed that Wor1 binding at the *WOR1* promoter is significantly decreased after just 1hr of temperature shift whereas Tup1 levels remain constant at the *WOR1* promoter (Figure 3.7B). This dramatic drop in relative levels of Wor1 to Tup1 at the *WOR1* promoter when shifting to 37°C may be the cause of the *en masse* opaque-to-white conversion. To address this, we depleted Tup1 from opaque cells at 37°C for 24hr and assessed opaque phase stability. While wild-type opaque cells converted completely to the white phase after shifting to 37°C, cells lacking Tup1 continued to express *WOR1* at 37°C and remained opaque upon re-plating on methionine-containing media (Figure 3.7C). These data suggest that loss of Wor1 binding at 37°C leads to Tup1 repression of *WOR1* expression and opaque-to-white switching, highlighting the centrality of Tup1 and providing a mechanism for temperature-induced phase switching.

Carbon source affects opaque stability and Tup1 level at the *WOR1* promoter

While high temperature drives conversion to the white state, opaque cells occur naturally within the human host, which has an ambient temperature of 37°C. How opaque cells are able to exist at this non-permissible temperature is not explained in the current model. Due to the niche specific optimization of *C. albicans*, we postulated that some external signal encountered in the host might stabilize the opaque state at 37°C. Opaque-like GUT cells are induced through passage in the mammalian GI tract, where glucose is low and alternative carbon sources such as GlcNAc and other derivatives of host and bacterial metabolism are present [20, 107]. In opaque cells, genes involved in glycolysis and glucose utilization are downregulated, while genes important in

respiration, gluconeogenesis, and fatty acid metabolism are upregulated [5]. Since opaque cells are metabolically hardwired to thrive in non-glucose conditions, we wondered if growth on alternative carbon sources might stabilize the opaque phase at high temperatures. We compared the effect of 12 different carbon sources in SC medium or SC alone, with amino acids as the sole carbon source, on opaque stability at 37°C for 24hr. We found that opaque cells cultured in the presence of the non-glycolytic carbon sources citrate, ethanol, lactate, glycerol, malic acid, succinic acid, and amino acids were able to maintain the opaque state. In contrast, opaque cells were not stabilized in the presence of glucose, maltose, raffinose, galactose, or fructose, which are all metabolized by glycolysis (Figure 3.8A). Since the stability assay was performed in SC media containing amino acids, which are non-glycolytic, the opaque state is destabilized by the presence of glycolytic carbon sources. As previously observed, GlcNAc was also able to stabilize opaque cells at 37°C despite being a glycolytic carbon source [10]. However, GlcNAc is a potent inducer of the opaque phase while the non-glycolytic carbon sources tested here do not induce opaque formation, so it is likely these carbon sources stabilize the opaque phase in a manner distinct from GlcNAc.

Tup1 has been shown to mediate glucose repression, and we find that both Tup1 depletion (Figure 3.7B) and growth in non-glycolytic carbon sources (Figure 3.8A) stabilize opaque cells at 37°C [93, 108]. Therefore, we hypothesized Tup1 binding at the *WOR1* promoter is probably reduced in cells cultured in non-glycolytic carbon sources. ChIP of Tup1 and Wor1 in opaque cells grown in glycerol and succinic acid at room temperature and 37°C show that Tup1 binding at the -4kb region of the *WOR1*

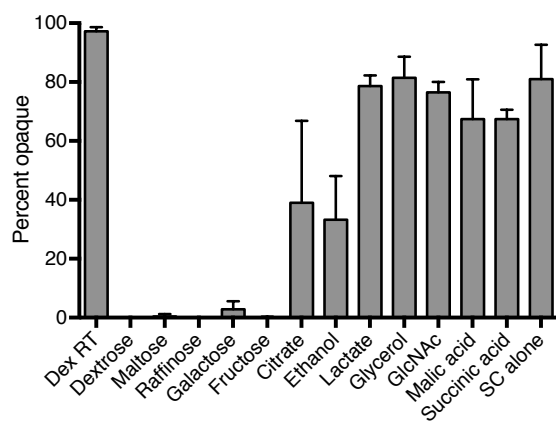
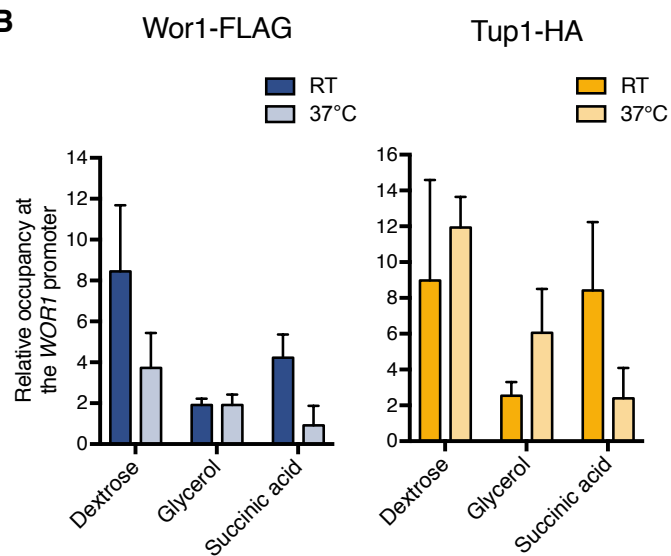
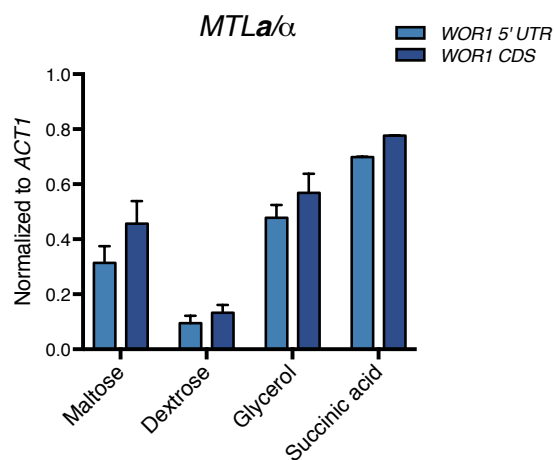
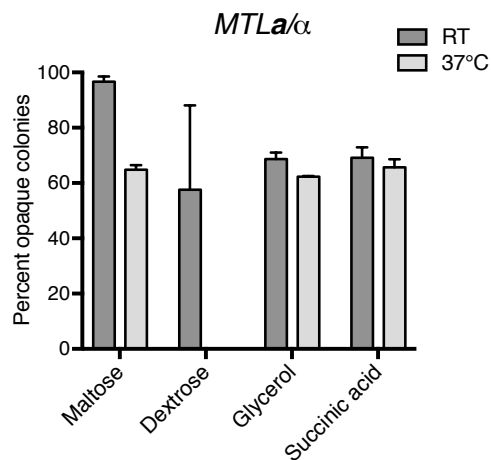
A**B****C****D**

Figure 3.8. Non-glycolytic carbon sources alter Tup1 occupancy at the *WOR1* promoter and stabilize the opaque phase at 37°C in *MTLa/a* and *a/a* cells. (A)

Opaque stability of *MTLa/a* cells cultured in various carbon sources at 37°C for 24hr. Overnight cultures of *MTLa/a* WT opaque cells (HLY3555) grown in SCD were washed three times with H₂O and inoculated into fresh SC medium containing the indicated carbon sources. Cultures were grown at room temperature for 3hr then transferred to 37°C for 24hr. Samples were plated onto SCD plates and grown for 5-7 days to assess phase switching. **(B)** ChIP of *Wor1* and *Tup1* in opaque cells at room temperature and 37°C in different carbon sources. Opaque cells carrying both *Wor1*-FLAG and *Tup1*-HA (HLY4541) and an untagged strain (JYC1) were grown in SC medium containing the indicated carbon source overnight at room temperature. Cultures were diluted and grown to log phase, then grown at either room temperature or 37°C for 1hr for ChIP. Enrichment is presented as a ratio of the -4kb region of the *WOR1* promoter IP (bound/input) over an *ADE2* control region IP (bound/input) of the tagged strain, further normalized to the control strain. Values are the average of three independent ChIP experiments with error bars representing the s.d. **(C)** Opaque stability of *MTLa/a* cells cultured in liquid media at 37°C for 24hr. Overnight cultures of opaque *MTLa/a* cells carrying *pMAL2-WOR1* (HLY4543) from SCM were washed three times with H₂O and inoculated into YNB medium containing the indicated carbon sources. Cultures were grown for 3hr at room temperature then shifted to 37°C. Cells were collected after 24hr and gene expression levels were analyzed by qPCR and normalized to *ACT1*. Average expression level of three independent qPCR experiments are plotted with error bars representing the s.d. **(D)** Opaque stability of *MTLa/a* cells on solid media. Overnight cultures of opaque *MTLa/a* cells carrying *pMAL2-WOR1* (HLY4543) grown in SCM were washed three times with H₂O then plated onto YNB plates containing 2% of the indicated carbon source. Plates were incubated at room temperature or 37°C for 5-7 days and scored for percent opaque. Both whole and sector opaque colonies were counted as opaque.

promoter is decreased compared to dextrose (Figure 3.8B). Interestingly, Wor1 binding was also decreased in these conditions, both at room temperature and 37°C (Figure 3.8B). The observed decrease in promoter occupancy was not due to global loss of Wor1 and Tup1, as Wor1 and Tup1 protein levels were not decreased when cultured in the presence of non-glycolytic carbon sources (Figure 3.9). Since opaque cells are stable in these culturing conditions, these data suggest that Tup1 level at the *WOR1* promoter is a driving force controlling opaque stability at 37°C. This reflects the importance of the relative levels and activities between the master activator Wor1 and the key repressor Tup1 at the *WOR1* promoter in opaque cell fate determination.

Non-glycolytic carbon sources stabilize the opaque phase in *MTLa/α* cells at 37°C.

Although most clinical isolates of *C. albicans* are *MTLa/α*, GUT cells are unstable upon exit from the mammalian host due to $\alpha 1$ - $\alpha 2$ repression of *WOR1* transcription [17, 18, 20-23]. The *in vitro* conditions that stabilize *MTLa/α* opaque cells outside of the mammalian gut are unknown. Since growth in alternative carbon sources was sufficient to stabilize opaque cells at 37°C in *MTLa/a* cells, we tested if it was also sufficient to stabilize the opaque phase in the presence of $\alpha 1$ - $\alpha 2$ repression. We transformed an *a/α* wild-type strain with *pMAL2-WOR1* to induce the opaque phase through ectopic expression of *WOR1* when cultured in maltose-containing YNB medium, which does not contain amino acids. Opaque *a/α* cells were washed and inoculated into YNB media containing different carbon sources, grown for 3hr to adapt to the carbon source, then shifted to 37°C for 24hr for expression analysis (Figure 3.8C). The *pMAL2-WOR1*

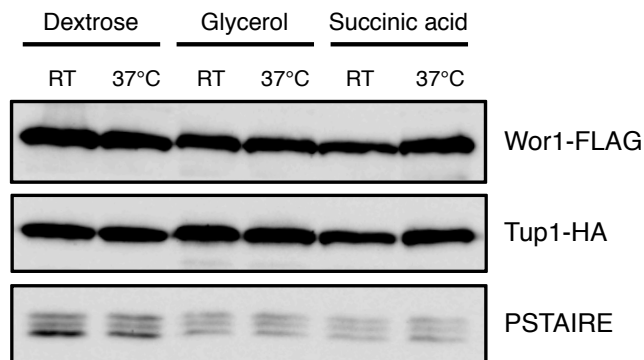


Figure 3.9. Protein levels of Wor1 and Tup1 remain constant when cultured in non-glycolytic carbon sources. Opaque cells carrying both Wor1-FLAG and Tup1-HA (HLY4541) were cultured overnight at room temperature in SC medium with the indicated carbon sources. Mid-log cultures were split then grown for an additional hour at either room temperature or 37°C and harvested. Tup1 and Wor1 levels were assessed by Western blot, as described.

construct only contains the *WOR1* coding sequence, allowing measurement of endogenous *WOR1* expression using primers specific to the *WOR1* 5' UTR. Due to ectopic *WOR1* expression from the *MAL2* promoter, cells grown in maltose were able to maintain endogenous *WOR1* expression, as indicated by qPCR of the 5' UTR. The higher level of *WOR1* CDS relative to 5' UTR likely reflects ectopic *WOR1* expression from the *MAL2* promoter. Conversely, cells grown in dextrose, known to repress the *MAL2* promoter, showed greatly reduced endogenous *WOR1* expression, evident by low transcript levels of *WOR1* 5' UTR and CDS. Importantly, cells cultured in glycerol and succinic acid were able to maintain high endogenous *WOR1* expression. The similar levels of 5' UTR and *WOR1* CDS indicate the absence of ectopic *WOR1* expression from the *MAL2* promoter. *WOR1* expression implied opaque stability, and this was confirmed on solid media. Using the *pMAL2-WOR1 MTL α / α* strain, cells induced to the opaque phase through ectopic *WOR1* expression were spread onto YNB plates containing different carbon sources and grown at 37°C (Figure 3.8D). Opaque cells on maltose plates remained opaque after 5-7 days of growth at 37°C due to maltose-induced ectopic *WOR1* overexpression. Opaque cells plated on glycerol and succinic acid were also able to maintain the opaque phase at 37°C, while cells plated on dextrose converted completely to the white phase (Figure 3.8D). Therefore, non-glycolytic carbon sources promote opaque stability at 37°C even in the presence of α 1- α 2 repression. This effect does not extend to all α 1- α 2 repressed genes, as opaque α / α cells are unable to mate [21].

DISCUSSION

Here we have identified Tup1, and Tup1 complex proteins as the major proteins associated with the master regulator Wor1. Our genetic analyses demonstrate that Tup1 is a key repressor of the opaque phase (Figure 3.10). Tup1 depletion and re-expression leads to complete white-to-opaque conversion, and importantly, Tup1 depletion is able to bypass the requirement for Wor1 in opaque phase establishment and maintenance. Therefore, we propose that Wor1 functions as the master regulator of the opaque switch by inhibiting Tup1-mediated repression of *WOR1* expression (Figure 3.10). We further show that levels of Wor1 or Tup1 at the *WOR1* promoter in opaque cells are regulated by temperature and carbon source, conditions that control opaque stability. We propose that external signals regulate Wor1 and Tup1 levels and activities at the *WOR1* promoter, and that these relative levels at the promoter control *WOR1* expression and determine cell fate (Figure 3.10). This study defines Wor1 and Tup1 as the central circuit for opaque phase transcription. Our model illustrates how external cues are interpreted to govern white-opaque switching.

The HBH tandem affinity purification system, utilizing first a Ni²⁺ purification followed by a streptavidin purification, is designed to purify proteins in denaturing conditions [71]. This system, combined with formaldehyde crosslinking, allows capturing of proteins with weak or transient interactions. Denaturing conditions used throughout the purification also reduce protein degradation and PTM loss. Indeed, the strength of this method was readily observed when we were unable to recapitulate the Wor1-Tup1 interaction by immunoprecipitation in native conditions absent of any cross-linkers. Ssn6, part of the Tup1 complex, was recently found to overlap with about 40 percent of Wor1-bound promoter regions [109]. The significant overlapping of Wor1 and Ssn6 on

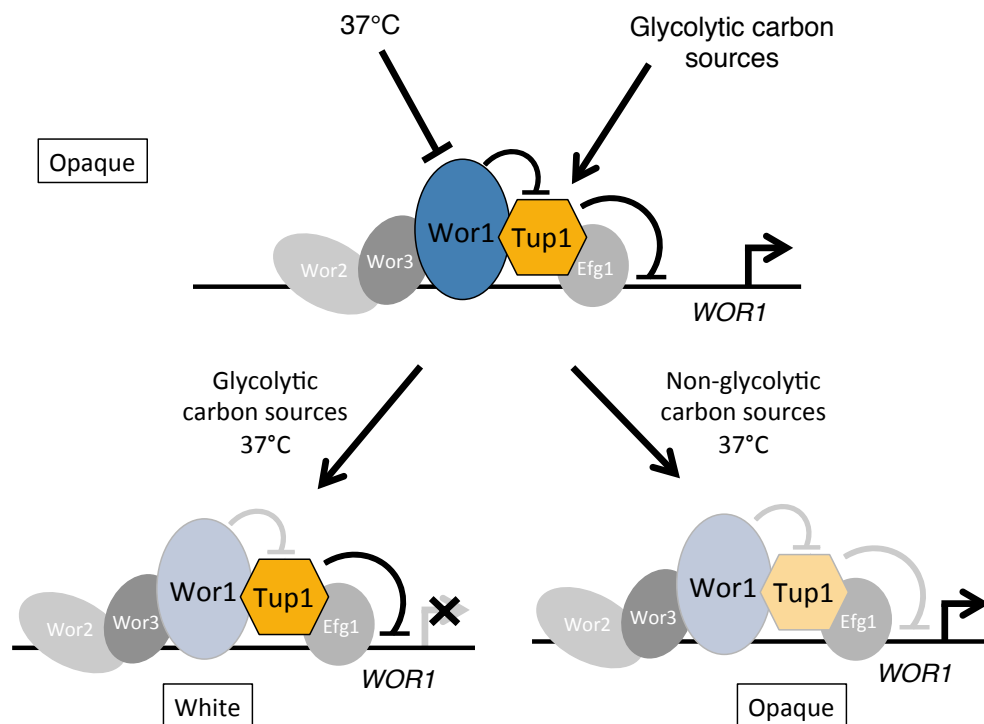


Figure 3.10. A model for Tup1-mediated regulation of cell fate at the *WOR1* promoter. Tup1 associates at the *WOR1* promoter and represses *WOR1* expression. In opaque cells, Wor1 binds along the *WOR1* promoter and inhibits Tup1-mediated repression of *WOR1* expression. The effect of temperature and carbon source on Wor1 and Tup1 occupancy at the *WOR1* promoter is shown. Dark blue or yellow indicates high levels of Wor1 and Tup1, while light colors depict decreased Wor1 or Tup1 at the promoter. Grey circles depict several other well-characterized phase regulators. Grey lines indicate reduced or lost activity.

promoter regions by ChIP-chip is consistent with Tup1, Ssn6 and Tcc1 being the major proteins found cross-linked to Wor1 in our purifications. However, the incomplete pattern of genome-wide co-occupancy of Wor1 and Ssn6 suggests that Wor1 does not recruit Ssn6/Tup1 to chromatin, as we would expect a more complete overlap of binding sites if that was the case. Instead, the Tup1 complex is likely recruited to different genomic regions by additional factors involved in the opaque circuit, such as Efg1, which co-occupies the same 2.4kb upstream region of the *WOR1* promoter as Tup1 in white cells. Interestingly, we did not identify Wor2 in our Wor1-HBH purifications, even though it has been shown to associate highly genome-wide with Wor1 by ChIP-chip [28]. This could be due to several reasons, including proximity to Wor1 on chromatin. So while HBH purification can be effective for identifying chromatin-mediated protein interactions, it is a method distinct from genome-wide ChIP. We believe it can be used to identify key cell fate regulators that function together in other systems.

A large body of work in the field has focused on the identification of new regulators of white-opaque switching, such as Efg1, Wor1, Wor2, Wor3, Czf1, and others [21-23, 28, 30, 31, 89, 109, 110]. Among these regulators, Wor1 is essential for opaque formation. Previous genetic studies have placed Czf1, Wor2 and Wor3 upstream of Wor1. Ectopic Wor1 expression bypasses *wor2* [31], *wor3* [89] and *czf1* [31] mutants in opaque formation, and overexpression of any of these regulators cannot bypass the *wor1* mutant [31, 89]. In contrast, Efg1 may function downstream of Wor1 as a *wor1 efg1* double mutant can become opaque under certain conditions [111]. Genome-wide ChIP studies of central transcription factors show they share similar opaque-enriched binding patterns along each other's promoter regions [28]. One

confounding finding, however, is that both positive and negative regulators, such as Efg1 which has reduced expression in opaque cells, show enriched promoter binding in opaque cells, notably at the 8kb-long, active *WOR1* promoter. Our biochemical approach identified Tup1, Ssn6, and Tcc1 as the major transcriptional regulators associated with Wor1. Wor3 and Efg1 were also found in our Wor1-HBH purifications, consistent with their overlapping occupancy at key opaque promoter regions with Wor1 (Figure 3.10). We further demonstrated that Tup1 is a key repressor of the opaque state. Tup1 depletion led to the expression of key opaque phase regulators, such as Wor1 and Wor3, but not of opaque-specific genes like *OP4*. Importantly, Tup1 depletion could bypass the Wor1 requirement for opaque phase formation. Recently, Ssn6 has also been identified as a repressor of the opaque cell type [109]. Unlike *tup1*, *ssn6* could not bypass *wor1* in opaque formation, likely due to a redundancy in function of the *Candida*-specific Ssn6 homolog Tcc1 [99]. As Tup1 does not bind DNA directly, it is likely recruited to promoter regions through interactions with other regulators, such as Efg1 (Figure 3.10). However, *efg1* cells can exist in the white state [112]. Therefore additional regulators are involved in recruiting Tup1 to repress key opaque circuit transcription. How Wor1 functions to inhibit Tup1-mediated repression of transcription is not clear. A similar phenomenon has been reported in *S. cerevisiae* regarding Tup1 repression of osmotic stress response genes, where Tup1 remains associated at active promoters upon stress activation [113]. Like Tup1 complexes, Taf14 was identified in all Wor1-HBH purifications and may play a role in transcription initiation and chromatin modification of the phase regulator genes [101, 114]. Our work presents an explanation by means of a previously unidentified function of Wor1 in inhibiting Tup1-mediated

repression. The central Wor1-Tup1 regulation allows for the integration of previously identified transcription factors in the white-opaque circuit, both activators and repressors, in controlling *WOR1* expression, with different regulators responding to different environmental cues. Multiple such regulations on the *WOR1* promoter ensure opaque stability, and transient alteration in expression of *WOR1* or other regulators is insufficient to convert opaque cells to the white phase.

A recent study has investigated the effects of a multitude of conditions on morphological programs [115]. Our study investigated two growth conditions, temperature and carbon source. Growth at 37°C converts opaque cells to white *en masse* in glucose-containing medium; correspondingly, Wor1 dissociates from the *WOR1* promoter while Tup1 level remains constant. Tup1 depletion and growth in non-glycolytic carbon sources both stabilize the opaque phase even at 37°C; correspondingly, Tup1 level at the *WOR1* promoter decreases in non-glycolytic carbon sources. We suggest that these two conditions are being integrated at the promoter chromatin to regulate the ratio of Wor1 and Tup1 level (and activity) at the *WOR1* promoter to control *WOR1* transcription and commitment to the opaque state. Temperature-dependent Wor1 binding could be regulated by Hsf1 and Hsp90 orchestrated chromatin remodelling [116]. Tup1 has previously been linked to regulation of carbon metabolism [93], and this and other glucose sensing pathways may control Tup1 occupancy or activity at the *WOR1* promoter. Multiple pathways are expected considering the observed differences in promoter-associated Tup1 and Wor1 between glycerol and succinic acid. The mechanistic details of these regulations require future experiments. One important finding is that lack of glycolytic carbon sources can

maintain opaque stability at 37°C even in *MTLa/α* cells where *WOR1* expression is repressed by $\alpha 1$ - $\alpha 2$. The capacity of alternative, non-glycolytic carbon sources to stabilize opaque *MTLa/α* cells at the physiologically relevant temperature of 37°C may explain the ability of opaque or GUT cells to exist in the host gastrointestinal tract, where glucose is low and non-glycolytic carbon sources are present in abundance. Additionally, growth on non-glycolytic carbon sources confers increased resistance to antifungals and other stresses [117] as well as increased virulence both *in vitro* and *in vivo* [117-119]. Therefore, the link between metabolism and cell state is of keen interest, and our work provides one mechanism by which carbon source and cellular metabolism can be sensed and integrated to contribute to the regulation of cell fate.

Both *Wor1* and *Tup1* are highly conserved fungal regulators. *Tup1* regulates hundreds of genes in *C. albicans* [93, 120] and has also been implicated in regulating metabolism, stress response, dimorphism, and virulence in a diverse range of pathogenic fungi, including the plant pathogens *Ustilago maydis* and *Magnaporthe oryzae* as well as the clinically relevant human pathogens *Aspergillus fumigatus* and *Cryptococcus neoformans* [121-124]. *Wor1* homologs in other fungi have been found to regulate developmental programs such as filamentation, sporulation, and virulence [34, 35, 125]. In *C. albicans*, *Tup1* is a repressor of both hyphal development and the opaque state. Here we show that *Wor1* functions as the master regulator of opaque cell fate by inhibiting *Tup1* repression to induce *WOR1* expression and the opaque phase. The same *Wor1*-*Tup1* regulatory circuit may be used widely in other fungi. In *C. neoformans* *Wor1*-homolog *Liv3* and *CnTup1* are both shown to regulate a gene required for quorum sensing and virulence [124, 126]. In *U. maydis*, *UmTup1* represses

the expression of the Wor1-homolog Pac2, and both proteins regulate filamentation and virulence. Importantly the *U. maydis tup1 pac2* double mutant resembles the *tup1* single mutant in gene expression and pathogenic phenotype [121]. These examples support the idea that the Wor1-Tup1 regulatory circuit is conserved across diverse fungi.

The content of this chapter was published in PLOS Genetics and can be referenced as follows:

Alkafeef SS, Yu C, Huang L, Liu H. Wor1 establishes opaque cell fate through inhibition of the general co-repressor Tup1 in *Candida albicans*. PLoS Genet. 2018;14(1):e1007176. doi: 10.1371/journal.pgen.1007176. PubMed PMID: 29337983.

CHAPTER 4

The glutaredoxin Grx3 is a global iron sensor and regulator of multiple iron-sulfur cluster-dependent pathways in *Candida albicans*

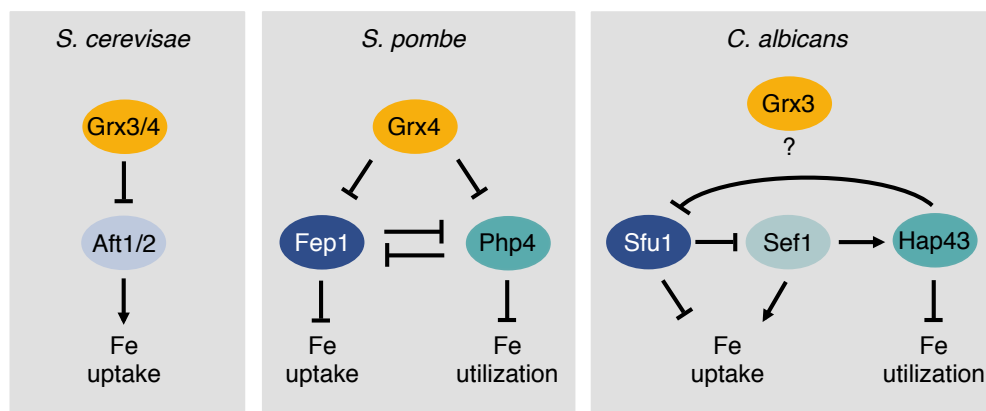
INTRODUCTION

Iron is an essential molecule that functions as a cofactor for many biological processes including respiration, metabolism, and DNA synthesis and repair. Lack of sufficient intracellular iron level and the cell is unable to perform the essential cellular functions required for life. Conversely, excess intracellular iron can also be toxic as it can lead to the generation of reactive oxygen species (ROS) via the Fenton reaction [37, 38, 127]. For pathogens, bioavailable iron level is dictated by host environment. *Candida albicans*, a fungal pathogen of humans, is characterized by its ability to inhabit a variety of distinct host niches encompassing a wide range of environmental iron bioavailability, including the bloodstream where most host iron is sequestered in the form of hemoglobin and transferrin, and the mammalian gastrointestinal tract, where iron levels are high as a result of dietary consumption [38, 50]. Therefore, the ability to regulate iron acquisition and utilization in response to environmental iron level is critical for *C. albicans* pathogenesis and survival. To that end, *C. albicans* utilizes a conserved transcriptional regulatory framework to maintain iron homeostasis (Figure 4.1A). In iron-replete conditions, the highly conserved GATA family transcription factor Sfu1 directly

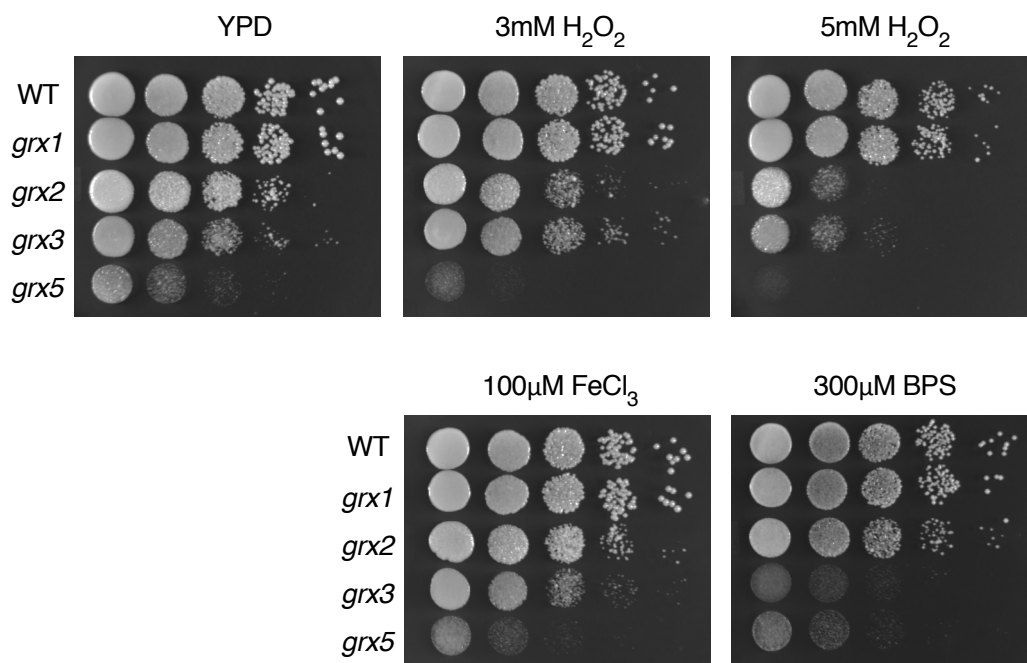
represses the expression of iron uptake genes as well as the expression of the Cys₆Zn₂ transcription factor Sef1, an activator of iron uptake gene expression [51, 57, 128, 129]. Therefore, Sfu1 is essential for *C. albicans* avoidance of iron toxicity and survival in iron-rich environments. Conversely, in low iron conditions, Sef1 induces the expression of the highly conserved CCAAT binding protein (CBP) transcription factor Hap43, which represses the expression of Sfu1 as well as the expression of nonessential iron utilization genes, increasing the intracellular iron pool and ensuring survival during iron limitation [51, 128-130]. While the roles of Sfu1 and Hap43 on the regulation of iron homeostasis are highly conserved across a broad range of fungi including *S. pombe* [68, 131-133], *H. capsulatum* [55, 56], and *C. neoformans* [53, 54, 134], *C. albicans* and several other *Candida* species are unique in their incorporation of Sef1 into the iron response regulon (Figure 4.1A) [51]. *In vivo* models have confirmed the essentiality of the *C. albicans* iron regulatory circuit on pathogenesis and fitness in the mammalian host [51, 129, 135]. However, the mechanism by which *C. albicans* senses iron availability and signals the iron regulatory circuit is unknown.

One class of proteins that have been implicated in iron sensing and homeostasis are the monothiol glutaredoxins (Grxs). Found in nearly all life forms ranging from bacteria to mammals, Grxs along with the functionally similar thioredoxins (Trxs) comprise a large family of thiol-disulfide oxidoreductases that regulate the redox state of cellular proteins [59-61]. Grxs can be categorized as dithiol (Class I) or monothiol (Class II) depending on their active site motifs and can display mitochondrial or cytosolic localization dependent on the specific Grx [59-61]. Both mono- and dithiol Grxs utilize

A



B



C

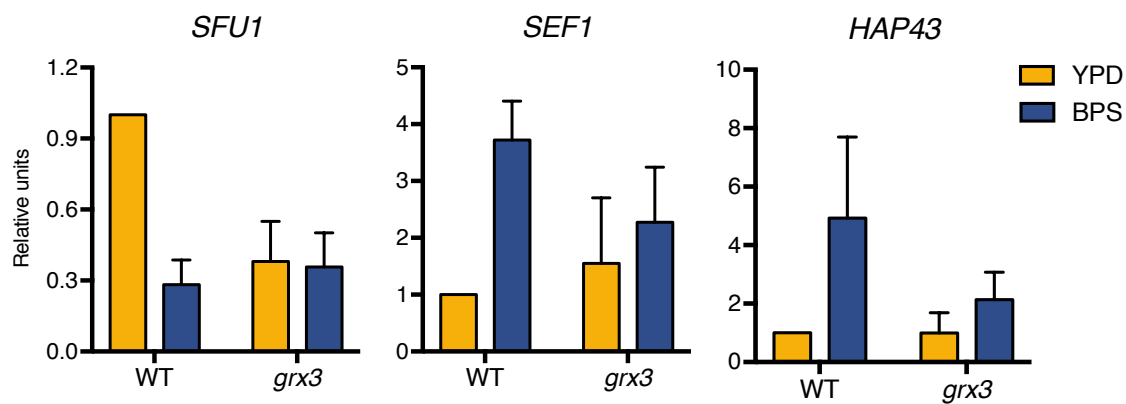


Figure 4.1. Grx3 is necessary for iron responsive regulation of the iron homeostasis circuit. **A.** Current models of iron homeostasis regulation in *S. cerevisiae*, *S. pombe*, and *C. albicans*. **B.** Spot tests of glutaredoxin mutants on oxidative and iron stresses. Wild-type (WT) (HLY4494), *grx1* (HLY4490), *grx2* (HLY4491), *grx3* (HLY4492), and *grx5* (HLY4493) were spotted with serial dilutions onto YPD plates containing either 3mM H₂O₂, 5mM H₂O₂, 100μM FeCl₃, or 300μM BPS and grown for 2-3 days at 30°C. **C.** Gene expression in WT and *grx3* mutant. WT (HLY4494) and *grx3* (HLY4492) cells were grown in YPD media in the presence or absence of 500μM BPS. Expression level of the indicated genes was measured by qPCR and normalized to *ACT1*. Presented is the average expression level from three independent qPCR experiments with error bars representing the s.d.

glutathione (GSH) and NADPH in redox reactions, but monothiol Grxs display little if any oxidoreductase activity *in vitro* [59, 61, 64]. Instead, these monothiol Grxs are able to bind iron-sulfur (Fe-S) clusters and are important for Fe-S cluster biogenesis and trafficking. Fe-S clusters are cofactors that play critical roles in the regulation of a multitude of biological processes [65]. The mitochondrial monothiol glutaredoxin Grx5 in *S. cerevisiae* plays a critical role in early Fe-S cluster biogenesis and is a core component of the early Fe-S cluster assembly (ISC) machinery necessary for the generation of Fe-S clusters. Disruption of Fe-S cluster biogenesis has been linked to diseases such as Friedreich ataxia, anemia, and dysfunctional erythropoiesis [66]. Cytosolic monothiol Grxs have been implicated in the trafficking and delivery of mature Fe-S clusters to client proteins, regulating the function of downstream processes dependent on Fe-S cofactors for activity [59-61]. In addition to their roles in redox homeostasis and Fe-S cluster biogenesis, monothiol Grxs have been shown to play critical roles in iron sensing and homeostasis in fungi, plants, and mammals [59-61]. In *S. cerevisiae*, under iron-replete conditions Grx3/4 form an Fe-S cluster bridge with the BolA-like proteins Fra1 and Fra2 [136, 137]. When assembled, this complex tethers the iron response transcriptional activators Aft1 and Aft2 to the cytosol, preventing the expression of iron uptake genes (Figure 4.1A). Decrease in Fe-S cluster due to iron limitation prevents formation of the Grx3/4-Fra1/2 complex and Aft1/2 translocates to the nucleus, inducing the expression of iron uptake genes [67]. In *S. pombe*, Grx4 acts on the iron regulatory circuit in two ways (Figure 4.1A). In the presence of iron, Fe-S cluster-carrying Grx4 traps Php4, a Hap43 homolog, in the cytosol, relieving repression

of iron utilization genes; upon iron limitation and a decrease in Fe-S cluster presence, the Grx4-Php4 complex dissociates and active Php4 translocates to the nucleus to repress gene expression [70]. Fe-S cluster-bound Grx4 is also present in the nucleus where it binds to Fep1 (an Sfu1 homolog), and is essential for Fep1-mediated repression of iron uptake genes [138, 139]. During iron limitation Fep1 donates its iron molecule to Grx4 in a reverse iron transfer mechanism, resulting in Fep1 dissociation from target promoters and loss of Fep1-mediated repression of iron uptake genes [52]. Whether monothiol Grxs play similar roles in iron sensing and homeostasis in *C. albicans* is yet to be determined.

Here we demonstrate that the conserved *C. albicans* cytosolic monothiol glutaredoxin Grx3 is essential for *C. albicans* response to iron levels, and regulates the iron homeostasis regulon on multiple levels. Using a mass spectrometry-based approach we identify over 300 Grx3 targets that function in diverse biological processes, including Fe-S biogenesis and iron homeostasis, redox homeostasis, metabolism, protein translation, and DNA synthesis and repair. This is the first genome-wide proteomic approach to identify Grx3 targets. The broadness of Grx3 targets signifies the importance of Grx3 in linking cellular iron levels to a diverse array of biological processes.

RESULTS

***C. albicans* grx3 mutant is sensitive to iron depletion**

Having previously been implicated in iron sensing in other fungi, we investigated the role of glutaredoxins (Grx) in the *C. albicans* iron response. *C. albicans* possesses at least four Grx family proteins: the Class I dithiol glutaredoxins Grx1 and Grx2 and the Class II monothiol glutaredoxins Grx3 and Grx5. While Grx1 and Grx5 remain largely uncharacterized, Grx2 and Grx3 have previously been implicated in oxidative stress response [140-142], consistent with the predicted functions of glutaredoxins in other organisms [59-61]. To distinguish the functions of the different *C. albicans* glutaredoxins, we examined the effect of oxidative stress on the viability of *grx* mutants. Cells of *grx1*, *grx2*, *grx3*, and *grx5* were spotted onto YPD plates in the presence and absence of the oxidative stress H_2O_2 (Figure 4.1B, top panels). As previously described, the *grx3* mutant was slightly defective on YPD plates [141], but the *grx5* mutant showed an even more severe growth defect on YPD (Figure 4.1B, top panels). The *grx2* mutant showed sensitivity to 3mM H_2O_2 , consistent with its identified role in oxidative stress response (Figure 4.1B, top panels) [140, 142]. At 5mM H_2O_2 , *grx2*, *grx3* and *grx5* all showed strong sensitivity, and *grx5* completely failed to grow in the presence of 5mM H_2O_2 (Figure 4.1B, top panels). *grx1* showed no enhanced sensitivity to H_2O_2 . These data illustrate the roles of *C. albicans* glutaredoxins in the oxidative stress response.

Since monothiol glutaredoxins are known to play important roles in Fe-S cluster biogenesis and transfer, we tested the *grx* mutants for sensitivity to iron stress (Figure 4.1B, bottom panels). To simulate a low-iron environment, we spotted cells onto YPD plates containing the iron chelator bathophenanthroline disulfonic acid (BPS). The *grx3* mutant showed a strong sensitivity to low iron stress when spotted onto BPS-containing

plates (Figure 4.1B, bottom panel). In contrast the *grx1*, *grx2* and *grx5* mutants showed no additional growth defect on BPS-containing plates. None of the mutants showed sensitivity to high iron stress in the form of excess FeCl_3 (Figure 4.1B, bottom panel). While *grx5* was defective in growth compared to wild-type even on YPD, this defect was not significantly enhanced by the addition of BPS (Figure 4.1B). Therefore, of the *C. albicans* glutaredoxin mutants tested, only the *grx3* mutant was sensitive to low iron level (Figure 4.1B). As work in *S. cerevisiae* [136] and *S. pombe* [70, 138] has implicated their respective Grx3 homologs in sensing and responding to intracellular iron level, we propose to determine if Grx3 also functions as an iron sensor in *C. albicans*.

Grx3 is required for iron-responsive expression of the iron homeostasis circuit genes

We next examined the role of Grx3 in the regulation of the iron homeostasis transcriptional circuit (Fig. 1A) [51]. In wild-type cells, *SFU1* is expressed in iron replete conditions but down regulated through the action of Hap43 during iron limitation [51]. Consistent with this, we observed that wild-type cells downregulated *SFU1* expression in the presence of BPS (Figure 4.1C). In accord with this observation, *SEF1*, which is transcriptionally repressed by Sfu1, and *HAP43*, which is transcriptionally induced by Sef1, were upregulated in response to BPS treatment (Figure 4.1C) [51]. Conversely, in the *grx3* mutant *SFU1*, *SEF1*, and *HAP43* expression were relatively unchanged in response to BPS treatment, suggesting that their expression level was no longer regulated in response to iron level in the absence of *grx3* (Figure 4.1C). Therefore, Grx3

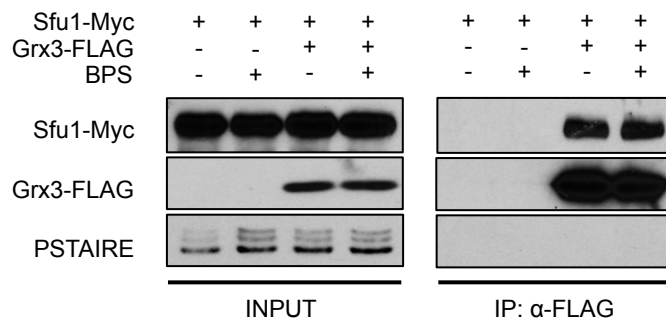
is important in sensing iron status and integrating this signal into the regulation of the *C. albicans* iron homeostasis regulatory circuit.

Grx3 interacts with Sfu1 and is necessary for Sfu1 association with target promoters

Since Grx3 is important for sensing intracellular iron level, we next examined how Grx3 is able to transmit this signal to control the iron homeostasis regulatory circuit. In *S. pombe*, the glutaredoxin Grx4 directly interacts with Fep1, an Sfu1 homolog, in both iron replete and iron limited conditions to regulate Fep1 occupancy at its target promoters [52, 69, 138]. To determine if Grx3 interacts with and regulates Sfu1 in a similar manner in *C. albicans*, we constructed a strain carrying both Grx3-FLAG and Sfu1-Myc, and examined their interaction in *C. albicans*. Due to the transcriptional regulation of *SFU1* in response to iron level, we placed the Sfu1-Myc construct under control of the *MAL2* promoter to ensure equal expression in both high and low iron conditions. Immunoprecipitation of Grx3-FLAG showed that Grx3 and Sfu1 interact *in vivo*, both in YPM (iron replete) and BPS-containing (iron limited) medium (Figure 4.2A). This interaction was unchanged by the addition of BPS and was equally strong in both high and low iron. Therefore, Grx3 and Sfu1 interact independent of iron level.

As Sfu1 binds to the *SEF1* promoter and represses its expression in iron replete conditions [51], we sought to determine if Grx3 regulates Sfu1 association with the *SEF1* promoter. Chromatin immunoprecipitation (ChIP) of maltose-induced Sfu1-Myc in wild-type cells revealed Sfu1 occupancy at the *SEF1* promoter was dependent on iron

A



B

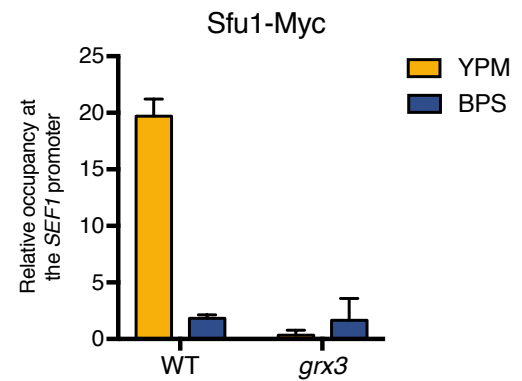


Figure 4.2. Grx3 interacts with Sfu1 to regulate its activity at the *SEF1* promoter. A. Immunoprecipitation of Grx3-FLAG. A strain carrying *pGRX3-GRX3-FLAG* and *pMAL2-SFU1-MYC* (HLY4500) and a control strain carrying just *pMAL2-SFU1-MYC* (HLY4498) were grown in the presence or absence of 500μM BPS at 30°C for 5hr before protein extraction and immunoprecipitation. **B.** Chromatin immunoprecipitation of Sfu1-Myc. WT (HLY4498) and *grx3* (HLY4507) strains carrying *pMAL2-SFU1-MYC* were diluted and grown in the presence or absence of 500μM BPS at 30°C for 5hr before formaldehyde crosslinking and protein extraction. Enrichment is presented as a ratio of qPCR of the *SEF1* promoter relative to an *ADE2* control region, with error bars representing SEM.

level (Figure 4.2B). In iron replete media (YPM) the *SEF1* promoter was highly bound by Sfu1, and this binding was lost upon the addition of BPS (low iron), consistent with previous observations (Figure 4.2B) [51]. The binding profile of Sfu1 at the *SEF1* promoter is also consistent with the transcriptional regulation of *SEF1* in high and low iron, with Sfu1 occupancy in iron-replete conditions correlating with *SEF1* repression (Figure 4.1C). Interestingly, in the *grx3* mutant, Sfu1 no longer bound the *SEF1* promoter in iron replete conditions (Figure 4.2B). This was also reflected in the transcriptional regulation of *SEF1* in the *grx3* mutant, where *SEF1* expression in *grx3* was higher than that of wild type cells in iron replete media (Figure 4.1C). Therefore, in iron replete conditions, Sfu1 occupies the *SEF1* promoter and represses expression in a Grx3-dependent manner. Our data suggests that Grx3 is required for Sfu1 to associate with its target promoters to repress gene expression.

Sfu1-independent regulation of the iron homeostasis circuit by Grx3

Having established that Grx3 regulates Sfu1 activity, we next assessed the role of Grx3 in the regulation of the other members of the iron homeostasis circuit, Sef1 and Hap43. To address if Grx3 regulates Sef1 and Hap43 in addition to its regulation of Sfu1, we constructed an *sfu1 grx3* double mutant and compared its phenotypes to that of the *grx3*, *sfu1*, *sef1*, and *hap43* single mutants in response to high and low iron stress (Figure 4.3A). If Grx3 functions solely through Sfu1 to regulate the iron homeostasis circuit, the *sfu1 grx3* double mutant should display a similar phenotype to that of the *sfu1* mutant. However, the *sfu1 grx3* double mutant behaved like *grx3* instead of *sfu1*

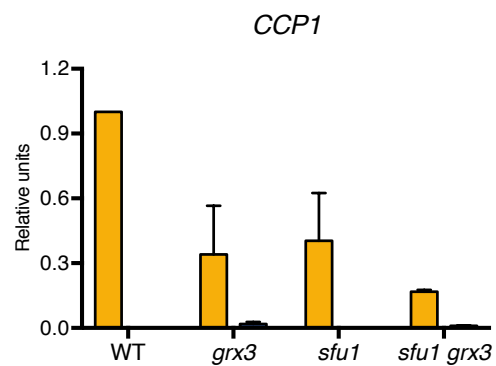
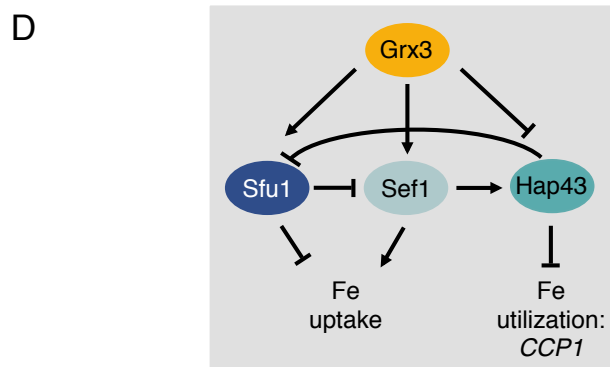
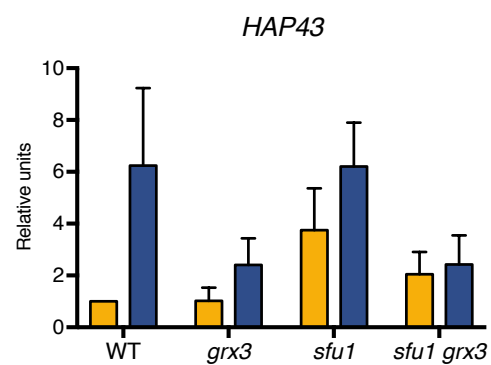
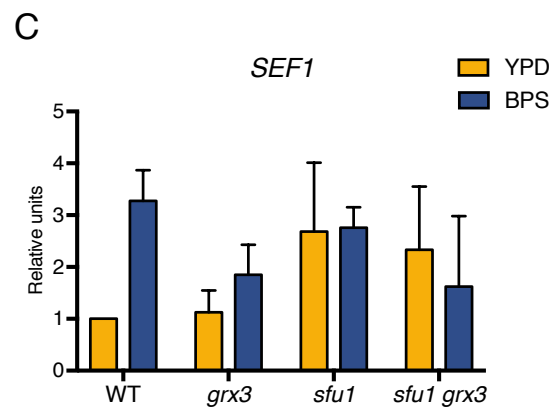
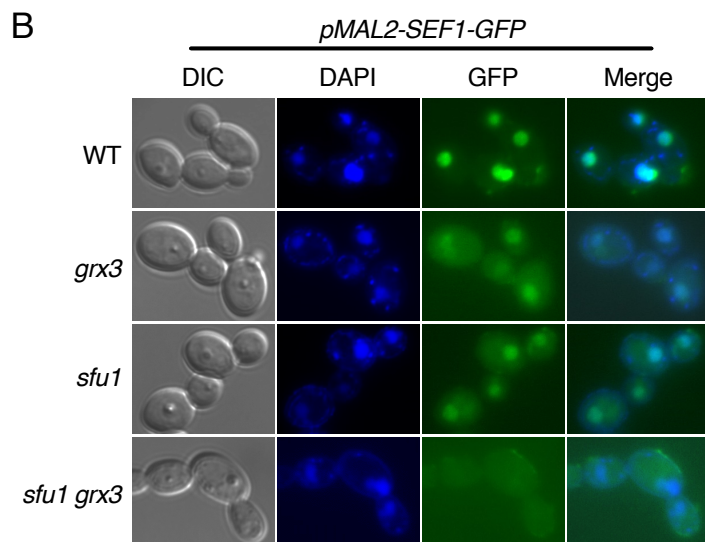
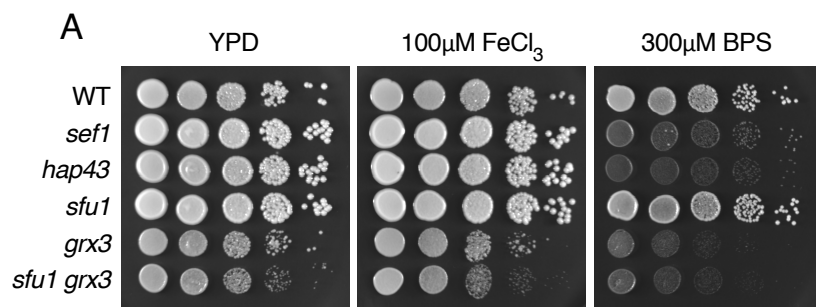


Figure 4.3. Grx3 regulates Sef1 localization and Hap43 activity. **A.** Spot tests of iron regulatory mutants on high and low iron plates. WT (HLY4494), *sef1*, *hap43*, *sfu1*, *grx3* (HLY4492), and *sfu1 grx3* (HLY4510) were spotted with serial dilutions onto YPD plates containing either 100μM FeCl₃ or 300μM BPS and grown at 30°C for 2-3 days. **B.** Localization of Sef1-GFP under control of the *MAL2* promoter. *pMAL2-SEF1-GFP* was transformed into WT (HLY4560), *grx3* (HLY4561), *sfu1* (HLY4562), and *sfu1 grx3* (HLY4563). Cells were grown in YPM with 500μM BPS for 6hr and Sef1-GFP localization was assessed by live cell imaging. Cells were stained with DAPI for nuclear visualization. **D.** Gene expression of *SEF1*, *HAP43* and *CCP1*. WT (HLY4494), *grx3* (HLY4492), *sfu1*, and *sfu1 grx3* (HLY4510) were grown in the presence or absence of 500μM BPS. Expression level was measured by qPCR and normalized to *ACT1*. Data is the average of three independent experiments with error bars representing the s.d.

(Figure 4.3A). Both the *grx3* single mutant and *sfu1 grx3* double mutant showed a similar growth defect on iron-limited media and resembled the *sef1* and *hap43* mutants, which are also unable to grow under iron limitation (Figure 4.3A) [51]. The fact that deletion of *sfu1* could not bypass the *grx3* phenotype suggests that Grx3 must contribute to the regulation of Sef1 and Hap43 in addition to its regulation of Sfu1 activity.

Grx3 is important for nuclear localization of Sef1

Sfu1 is known to repress Sef1 both transcriptionally and at the level of protein localization [57]. To determine if Grx3 regulates Sef1 independent of Sfu1, we examined Sef1 localization in the *grx3*, *sfu1* and *sfu1 grx3* mutants. To exclude the effects of transcriptional regulation of *SEF1* by Sfu1 in our assessment of the roles of Grx3 and Sfu1 on Sef1 localization, we generated a conditional Sef1-GFP reporter under the control of the strong *MAL2* promoter. Using the *pMAL2-SEF1-GFP* construct, we grew cells in YPM media containing BPS for 6hr to induce a low iron effect and examined Sef1-GFP localization (Figure 4.3B). In wild-type cells, Sef1-GFP localized mostly to the nucleus (Figure 4.3B). However, the *grx3* mutant displayed decreased Sef1-GFP nuclear localization with an increased cytosolic presence (Figure 4.3B). The *sfu1* mutant also showed an increased Sef1-GFP presence in the cytosol compared to wild-type (Figure 4.3B). Strikingly, the *sfu1 grx3* double mutant showed an even stronger defect in Sef1-GFP nuclear localization; no enrichment in GFP signal was observed in the nucleus over the cytosol (Figure 4.3B). Therefore, Grx3 and Sfu1 both contribute to the

regulation of Sef1 on two levels: through the transcriptional regulation of *SEF1* expression (Figs 1C, 2B) and through localization of Sef1 protein (Figure 4.3B). Sef1 is known to regulate *HAP43* expression in *C. albicans* [51]. We observed that *HAP43* expression is low in the *grx3* and *sfu1 grx3* mutants under iron depletion conditions, further supporting the role of Grx3 in Sef1 activity (Figure 3C).

Grx3 regulates Hap43 activity

We next turned our attention to the role of Grx3 on Hap43 function as Grx4 has been shown to regulate the Hap43 homolog Php4 in response to iron level in *S. pombe* [52, 70, 143]. To assess Hap43 activity, we measured expression of the iron utilization gene *CCP1*, a putative cytochrome c oxidase, in iron replete and iron limited conditions (Figure 4.3C). Of the three iron circuit regulators, only Hap43 binds to the *CCP1* promoter, where it functions to repress *CCP1* expression in low iron conditions, making *CCP1* an ideal gene to assay Hap43 activity. In wild-type cells, *CCP1* was expressed in YPD but strongly repressed in BPS-containing media (Figure 4.3C). Consistent with this, the *sfu1* mutant showed increased *HAP43* expression and decreased *CCP1* expression in YPD compared to wild-type (Figure 4.3C). In contrast, in the *grx3* mutant, *HAP43* level was similar to that of wild-type, but *CCP1* expression decreased compared to wild-type, suggesting that Hap43 activity maybe higher in the *grx3* mutant (Figure 4.3C). In further support of this notion, deletion of *GRX3* in the *sfu1* mutant decreased levels of *HAP43* expression as well as *CCP1* expression. The *sfu1 grx3* double mutant showed a greater decrease in *CCP1* expression compared to the *sfu1* or *grx3* single

mutants (Figure 4.3C). The additive effect of the loss of both *sfu1* and *grx3* suggests that, consistent with our earlier observations with Sef1 nuclear localization (Figure 4.3B), Grx3 and Sfu1 have independent roles in the regulation of Sef1 and Hap43 at the level of protein activity (Figure 4.3D). In addition to *CCP1*, Hap43 also represses *SFU1* expression. Like *CCP1*, *SFU1* expression level in the *grx3* mutant in YPD was lower compared to wild-type (Figure 4.1C), indicating increased Hap43 activity in the absence of Grx3. Our data suggests that Grx3 represses Hap43 activity (Figure 4.3D). This is consistent with work in *S. pombe*, where Grx4 has been shown to regulate Php4 activity in response to iron level. In high iron Grx4 interacts with and sequesters Php4 in the cytosol but upon iron limitation this interaction is disrupted and Php4 translocates to the nucleus where it represses iron utilization gene expression [52, 70, 143].

Identification of Grx3 interacting proteins by a proteomic approach

Having shown that Grx3 regulates the iron homeostasis circuit proteins Sfu1, Sef1 and Hap43, we next wanted to determine what other proteins and processes Grx3 may regulate. We utilized cross-linked tandem affinity purification in denaturing conditions coupled with mass spectrometry using a His-Biotin-His (HBH) tag [71, 80] to identify Grx3 interacting protein targets *in vivo*. A *pMAL2*-driven Grx3-HBH fusion construct was generated and transformed into the *grx3* mutant strain. The fusion construct was functional, as growth on maltose medium was capable of rescuing the *grx3* phenotype when cultured on BPS-containing plates (Figure 4.4). The HBH tag utilizes two high affinity purification strategies that are functional in denaturing

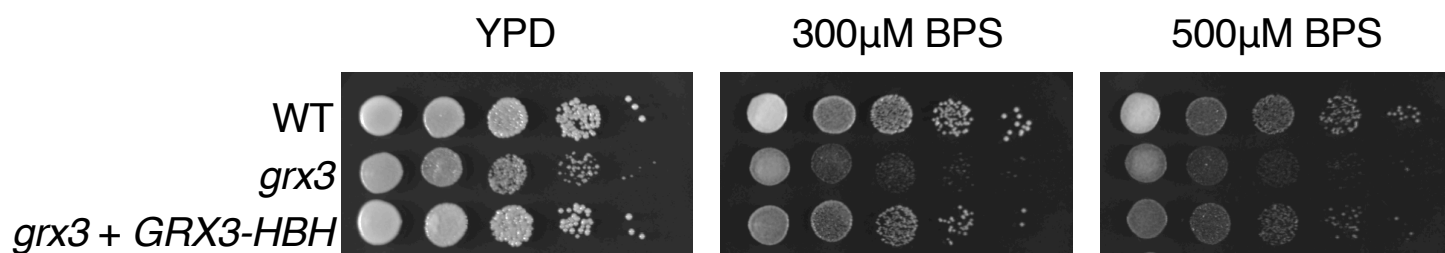


Figure 4.4. The Grx3-HBH tag is functional. WT (HLY4494), *grx3* (HLY4492), and *grx3* transformed with *pMAL2-GRX3-HBH* (HLY4559) were spotted onto YPM plates with 300μM or 500μM BPS and grown at 30°C for 2-3 days.

conditions. Performing the purification under denaturing conditions helps prevent loss of post-translational modifications and reduces the occurrence of protein degradation. Additionally, formaldehyde cross-linking helps preserve weak or transient interactions during cell lysis and the purification process. Using a control untagged wild-type strain and our Grx3-HBH fusion strain we diluted overnight cultures into fresh YPM in the presence or absence of 500 μ M BPS and grew them for 5hr at 30°C before formaldehyde fixation and lysis in an 8M urea denaturing buffer. Clarified protein extracts were incubated with Ni²⁺ beads, washed, eluted with imidazole and low pH, then incubated with streptavidin-conjugated beads. The samples were subjected to on-bead trypsin digestion and resulting peptides were analyzed by mass spectrometry. Any interacting proteins that appeared in the untagged control samples were excluded from analysis as they represent nonspecific interactions. We believe this method is ideally suited to identifying Grx3 interacting proteins for a number of reasons. Firstly, as an Fe-S cluster binding and delivery protein, Grx3 functions through direct interactions with Fe-S cluster recipient proteins. Secondly, since Grx3 is expected to contribute to a wide array of biological processes, a global proteomic approach might provide a more efficient and comprehensive method to identify critical Grx3 client proteins essential for the regulation of these processes.

We identified 310 putative Grx3-interacting proteins involved in wide array of biological processes and functions (Table 4.1). We did not observe any significant differences in Grx3 protein association in response to iron level, consistent with our previous observation that Grx3 interacts with Sfu1 regardless of iron level (Figure 4.2A).

Table 4.1: Grx3 interacting proteins identified by mass spectrometry

#	Gene Name	ORF	#	Gene Name	ORF	#	Gene Name	ORF
1	SSB1	orf19.6367	105	ACB1	orf19.7043.1	209	UCF1	orf19.1354
2	EFT2	orf19.5788	106	ZUO1	orf19.2709	210		orf19.2269
3	HSP70	orf19.4980	107	RPS18	orf19.7018	211		orf19.5016
4	SSA2	orf19.1065	108	RPL82	orf19.2311	212	RAD23	orf19.1494
5	MSI3	orf19.2435	109	TRR1	orf19.4290	213	NAS6	orf19.5961
6	PGK1	orf19.3651	110		orf19.1946	214		orf19.2737
7	TDH3	orf19.6814	111	VAS1	orf19.1295	215	RPP2B	orf19.5928
8	HSP90	orf19.6515	112	MDH1-1	orf19.4602	216	THS1	orf19.5685
9		orf19.2095	113		orf19.1707	217	PFK2	orf19.6540
10	ENO1	orf19.395	114	RPL11	orf19.2232	218	LHP1	orf19.2795
11	TAL1	orf19.4371	115	TMA19	orf19.3268	219	HPT1	orf19.5832
12	GRX3	orf19.2727	116	SSZ1	orf19.3812	220		orf19.7263
13	ADH2	orf19.5113	117	YNK1	orf19.4311	221		orf19.7069
14	MET6	orf19.2551	118	TUP1	orf19.6109	222		orf19.6705
15	CDC19	orf19.3575	119	UBA1	orf19.7438	223	GDI1	orf19.7261
16		orf19.2296	120	MLC1	orf19.2416.1	224	PAA11	orf19.7269
17	FBA1	orf19.4618	121	GSP1	orf19.5493	225	CCS1	orf19.4449
18	TIF	orf19.3324	122		orf19.6701	226		orf19.6559
19	GND1	orf19.5024	123	PRX1	orf19.5180	227		orf19.5054
20	CEF3	orf19.4152	124	PCK1	orf19.7514	228	GAD1	orf19.1153
21	ATP1	orf19.6854	125	RPS8A	orf19.6873	229	SOD3	orf19.7111.1
22	ALD5	orf19.5806	126	SGT1	orf19.4089	230	MDH1-3	orf19.5323
23	TSA1B	orf19.7398.1	127	WH11	orf19.3548.1	231	GLY1	orf19.986
24	KRS1	orf19.6749	128		orf19.5525	232	CRN1	orf19.6534.2
25	HKX2	orf19.542	129	RPS5	orf19.4336	233	SIS1	orf19.3861
26	PDC11	orf19.2877	130	RPL12	orf19.1635	234	EIF4E	orf19.7626
27	GPH1	orf19.7021	131	TIF5	orf19.4261	235	GFA1	orf19.1618
28	SHM2	orf19.5750	132	SUB2	orf19.5647	236	RPL24A	orf19.3789
29	CAM1	orf19.7382	133	HCR1	orf19.7613	237	ERG6	orf19.1631
30	CAM1-1	orf19.2651	134	ARO3	orf19.1517	238	UGP1	orf19.1738
31	SAH1	orf19.3911	135	GCD11	orf19.4223	239	PDB1	orf19.5294
32	GLX3	orf19.251	136	SOU1	orf19.2896	240	ASR3	orf19.842
33	BMH1	orf19.3014	137	PET9	orf19.930	241	ADE1	orf19.7484
34	DPS1-1	orf19.2407	138	RIB4	orf19.410.3	242	UBC1	orf19.1085
35	ERG13	orf19.7312	139	NHP6A	orf19.4623.3	243		orf19.6596
36	CAR2	orf19.5641	140		orf19.3053	244	AGE3	orf19.3683
37	ACT1	orf19.5007	141	PMI1	orf19.1390	245	SUI1	orf19.1280
38	RCT1	orf19.7350	142	EGD1	orf19.1154	246	GUK1	orf19.1115
39	IFR2	orf19.2396	143	AAT1	orf19.3554	247	GUA1	orf19.4813
40	XYL2	orf19.7676	144	SUP35	orf19.1378	248	KIS1	orf19.4084
41	MET18	orf19.1706	145	GRS1	orf19.437	249	LSC2	orf19.1860
42	AHP1	orf19.2762	146	ECM4	orf19.2613	250	SAC6	orf19.5544
43	SRB1	orf19.6190	147	SPE3	orf19.2250	251	VMA10	orf19.1866
44	PAB1	orf19.3037	148	RPS42	orf19.3354	252	CAR1	orf19.3934
45	CYP1	orf19.6472	149	TOM70	orf19.3700	253	SEC14	orf19.941
46	CPR6	orf19.7654	150	HAP2	orf19.1228	254	LAT1	orf19.6561

47	SES1	orf19.269	151	VMA4	orf19.2598	255	GSY1	orf19.3278
48	CSH1	orf19.4477	152	RPL10A	orf19.3465	256	SDS24	orf19.5118
49	IFD6	orf19.1048	153	RPS21B	orf19.3325.3	257	ACH1	orf19.3171
50	DAK2	orf19.4777	154	RPS28B	orf19.7048.1	258	IDH1	orf19.4826
51	IMH3	orf19.18	155	HMO1	orf19.6645	259	ARA1	orf19.2172
52	EGD2	orf19.5858	156	TUB2	orf19.6034	260	SUI3	orf19.7161
53	DDR48	orf19.4082	157	FUM12	orf19.6724	261	FDH1	orf19.638
54	GPM1	orf19.903	158	RPS17B	orf19.2329.1	262	UBC4	orf19.7571
55	ASN1	orf19.198	159	BCY1	orf19.2014	263	RPL19A	orf19.5904
56	GRP2	orf19.4309	160	TRX1	orf19.7611	264		orf19.7489.3
57	ABP1	orf19.2699	161	PIN3	orf19.5956	265	RPS9B	orf19.838.1
58	HSP12	orf19.3160	162	STI1	orf19.3192	266	RLI1	orf19.3034
59	MIS11	orf19.2364	163	GPX1	orf19.86	267	ETR1	orf19.5450
60	ZWF1	orf19.4754	164	AHP2	orf19.6470	268	PRT1	orf19.6584
61	YBN5	orf19.754	165	CDC48	orf19.2340	269	MVD	orf19.6105
62	PMM1	orf19.2937	166	AAT22	orf19.4669	270		orf19.5773
63	SLK19	orf19.6763	167	RPS3	orf19.6312	271	RPP0	orf19.7015
64	TFP1	orf19.1680	168	GDH2	orf19.2192	272	HET1	orf19.6327
65	TPI1	orf19.6745	169	PFY1	orf19.5076	273	MIA40	orf19.2977
66	SPX5	orf19.6507	170	IDI1	orf19.2775	274	YHB1	orf19.3707
67	TKL1	orf19.5112	171	HOM6	orf19.2951	275	RIB1	orf19.2862
68	EFB1	orf19.3838	172	YRB1	orf19.7477	276		orf19.3475
69	GAL1	orf19.3670	173	MAL32	orf19.3982	277	SMT3	orf19.670
70	ADE17	orf19.492	174	ARO4	orf19.4060	278	ALA1	orf19.5746
71	SAM2	orf19.657	175	TAF14	orf19.798	279		orf19.2769
72	BAT22	orf19.6994	176	MCR1	orf19.3507	280	SUI2	orf19.6213
73	ILS1	orf19.2138	177	MBF1	orf19.3294	281	KRE30	orf19.2183
74	PGI1	orf19.3888	178	RPS14B	orf19.6265.1	282	APT1	orf19.1448
75		orf19.5943.1	179	ERG20	orf19.4491	283	RHO1	orf19.2843
76	MDG1	orf19.7239	180	RPL23A	orf19.3504	284	PRE2	orf19.2233
77	CDC60	orf19.2560	181	ERF1	orf19.3541	285	PRE9	orf19.350
78	DED81	orf19.6702	182		orf19.4246	286	GLC7	orf19.6285
79	GRE3	orf19.4317	183	RPS6A	orf19.4660	287	RPL9B	orf19.236
80	COF1	orf19.953.1	184	DBP5	orf19.1661	288	LAP3	orf19.539
81	TIF3	orf19.3423	185	SHP1	orf19.2549	289	WRS1	orf19.5226
82	FDH3	orf19.7600	186	ADE12	orf19.4827	290	RPL20B	orf19.4632
83	ARC1	orf19.2422	187	CYC1	orf19.1770	291	ZRT2	orf19.1585
84	GLK1	orf19.734	188	RNR1	orf19.5779	292	RIB5	orf19.4024
85		orf19.3061.1	189	ARO2	orf19.1986	293	PMA1	orf19.5383
86	ERG10	orf19.1591	190	THR4	orf19.4233	294	DYS1	orf19.1626
87	APE2	orf19.5197	191	RPS7A	orf19.1700	295	TYS1	orf19.2694
88	ATP2	orf19.5653	192	SNF4	orf19.5768	296	HTS1	orf19.4051
89	TPM2	orf19.6414.3	193	HOM2	orf19.1559	297	RPS21	orf19.3334
90	YST1	orf19.6975	194	GLR1	orf19.4147	298		orf19.285
91		orf19.338	195	RBP1	orf19.6452	299	CCT3	orf19.4004
92	PST3	orf19.5285	196	PUP3	orf19.1336	300	DOT5	orf19.5417
93	ADO1	orf19.5591	197	HEM13	orf19.2803	301		orf19.6220.4
94	ARF2	orf19.5964	198		orf19.7214	302	TIM50	orf19.680
95	PIL1	orf19.778	199	PRB1	orf19.7196	303	SNF7	orf19.6040
96	IPP1	orf19.3590	200	RIB3	orf19.5228	304		orf19.3915
97	GUS1	orf19.7057	201	RPS12	orf19.6785	305	RPS10	orf19.2179.2

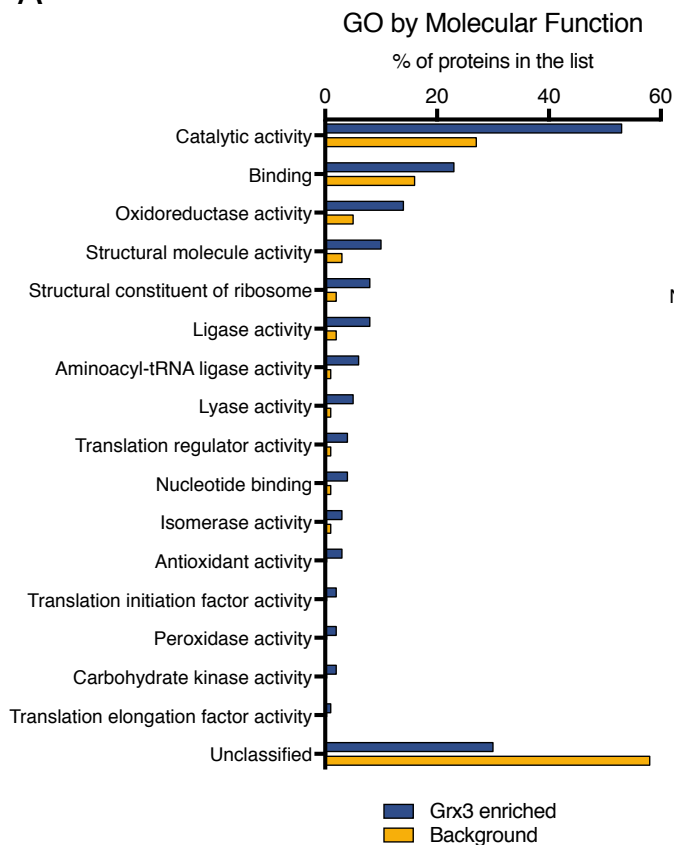
98	RNR21	orf19.5801	202	RNA1	orf19.1649	306	SGT2	orf19.5823
99	MAL2	orf19.7668	203	RHR2	orf19.5437	307	SLS1	orf19.6403
100	PGM2	orf19.2841	204	RPL43A	orf19.3942.1	308	LYS1	orf19.1789.1
101	RDI1	orf19.5968	205	GLN4	orf19.7064	309		orf19.1862
102	RPL4B	orf19.7217	206	RPL30	orf19.3788.1			
103	GLO3	orf19.5445	207	LYS9	orf19.7448			
104	ACO1	orf19.6385	208		orf19.4164			

Table 4.1. Grx3 interacting proteins identified by mass spectrometry. Overnight cultures of an untagged wild-type strain (HLY4494) and a *grx3* mutant strain carrying *pMAL2-GRX3-HBH* (HLY4559) were diluted into fresh YPM media and grown for 5hr at 30°C in the presence or absence of 500μM BPS. Proteins listed were identified in Grx3-HBH purifications performed under formaldehyde cross-linking conditions and did not appear in any untagged control strain samples. As no significant differences in Grx3 interacting proteins were observed between high and low iron treatment, data from both samples were combined and are represented here in order of decreasing abundance.

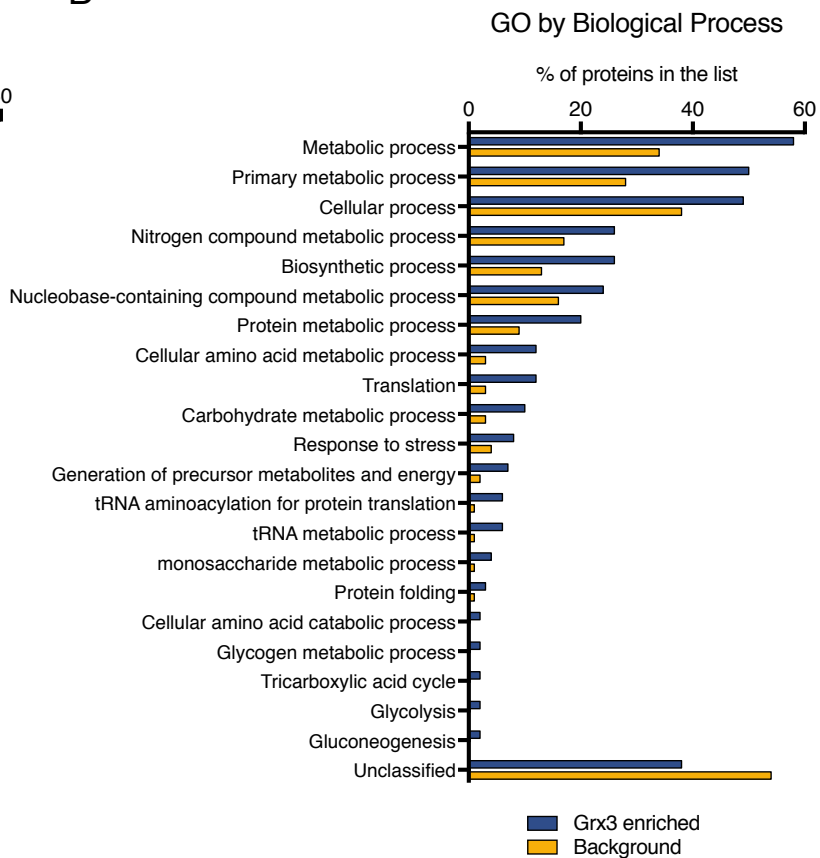
In our mass spectrometry data we identified a number of proteins previously shown in *S. cerevisiae* and *S. pombe* to interact with their respective Grx3 homologs or to bind and carry Fe-S clusters, such as the Fe-S cluster binding BolA-like proteins orf19.2095 (ScFra1), orf19.7489.3 (ScFra2), orf19.5016 (ScBol1) [61]. Work in *S. cerevisiae* and *S. pombe* has implicated Fra1 and Fra2 homologs in Grx-mediated regulation of their respective iron homeostasis regulons and also implicated BolA proteins in Fe-S cluster biogenesis and trafficking [61]. We also observed other proteins known to play roles in iron homeostasis or be directly regulated by iron including the mitochondrial disulfide relay protein Mia40 [144], the Fe-S cluster-dependent TCA cycle enzymes aconitase (Aco1) and fumarase (Fum12) [145-147], the CCAAT binding complex subunit Hap2 [129], and the heme biosynthesis protein Hem13 (Table 4.1) [148]. These observations of bona-fide Grx interacting proteins as well as other known iron binding proteins in our mass spectrometry data validate our proteomic method and support the role of Grx3 in regulating *C. albicans* Fe-S cluster biogenesis, trafficking, and iron homeostasis [136, 137, 139, 149]. To the best of our knowledge, this is the first comprehensive proteomic approach undertaken to identify Grx3 associated proteins *in vivo*.

Gene Ontology (GO) analysis was performed using the PANTHER Classification System [150, 151]. Of the 310 identified proteins, 270 mapped to reference classifications. By GO Slim analysis of molecular function, Grx3 interacting proteins fell into several distinctive categories including oxidoreductase, peroxidase, and antioxidant activity, consistent with the known involvement of Grx3 in maintaining cellular redox homeostasis (Figure 4.5A) [141]. Interestingly, catalytic activity showed the highest

A



B



C

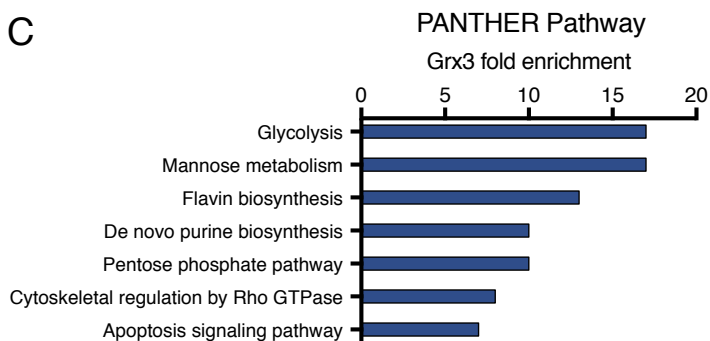


Figure 4.5. Grx3 interacting proteins display diverse functions and contribute to a wide array of biological processes. Gene Ontology analysis of Grx3 interacting proteins identified by mass spectrometry. **(A)** Proteins were analyzed using the PANTHER GO Slim Molecular Function, **(B)** PANTHER GO Slim Biological Process, and **(C)** PANTHER Pathways. Results with a FDR of > 0.05 were excluded.

enrichment, suggesting that function of many Grx3 interacting proteins may depend on the presence of Fe-S clusters, cofactors carried and trafficked by glutaredoxins like Grx3 [59]. Many putative Grx3 interacting proteins showed molecular functions related to translation, such as ribosomal proteins, translation regulator and initiation factor activity, and tRNA ligase activity (Figure 4.5A).

GO Slim analysis by biological process confirmed our molecular function GO analysis, and we observed enrichment in our dataset for processes such as stress response, translation, tRNA metabolism, and tRNA aminoacylation (Figure 4.5B). We also observed enrichment in various metabolic and biosynthetic processes including amino acid metabolism, carbohydrate metabolism, generation of precursor metabolites, the TCA cycle, glycolysis, and gluconeogenesis (Figure 4.5B). Categorized under protein folding, our mass spectrometry data set also identified several heat shock proteins to interact with Grx3 (Figure 4.5B). In addition to confirming enrichment of Grx3 interacting proteins in metabolic and biosynthetic processes as in Figure 4.5A and Figure 4.5B, PANTHER pathways analysis revealed enrichment of specific pathways, such as flavin biosynthesis, *de novo* purine biosynthesis, the pentose phosphate pathway, and apoptosis signaling (predominantly represented by heat shock proteins) (Figure 4.5C). Therefore, Grx3 plays a broad role in the regulation of multiple biological processes. In other species, Grx3 homologs are known to function as Fe-S carriers that deliver Fe-S clusters to proteins whose functions are dependent on the essential cofactor. Since many of our Grx3 interacting proteins enriched for processes known to be functionally dependent on Fe-S clusters such as DNA maintenance, protein

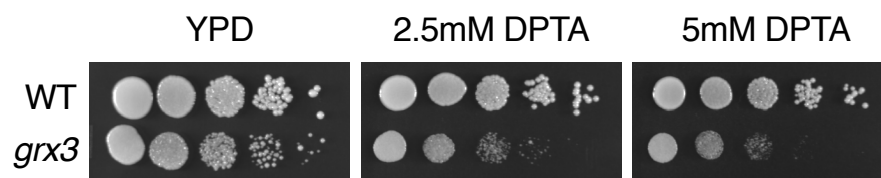
translation, and metabolism [65, 127, 152-154], we propose that *C. albicans* Grx3 functions as a global iron sensor and Fe-S cluster carrier that regulates multiple pathways through cluster trafficking to destination proteins involved in a wide array of biological processes.

Grx3 is important for multiple iron-sulfur cluster-dependent processes

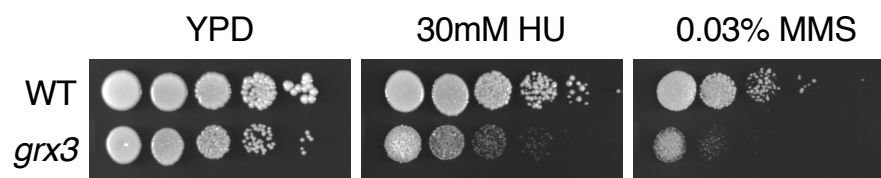
We next sought to experimentally assess and validate the role of Grx3 in the function of different processes enriched by GO analysis of our mass spectrometry data (Figure 4.5, Table 4.1). We observed GO enrichment of several cellular stress response categories such as oxidoreductase, antioxidant, and peroxidase activities (Figure 4.5A). Having shown Grx3 sensitivity to oxidative stress (Figure 4.1B) and having observed enrichment of nitrogen compound metabolic processes in our GO analysis (Figure 4.5B), we tested the affect of nitrosative stress on the *grx3* mutant. Spotting wild-type and *grx3* cells onto YPD plates containing the nitrosative stress DPTA NONOate, we observed an increased sensitivity in the *grx3* mutant, confirming our GO enrichment data and extending the role of Grx3-mediated stress response to include nitrosative stress (Figure 4.6A).

Our mass spectrometry data also indicated that Grx3 might play a role in maintaining genomic integrity. GO analysis showed enrichment in nucleotide binding and biosynthesis proteins (Figure 4.5), and Fe-S cluster biogenesis has been implicated in DNA replication and repair pathways [155]. Additionally, we found Grx3 to interact with the DNA damage response and DNA replication proteins Rad23, Met18, Rnr21,

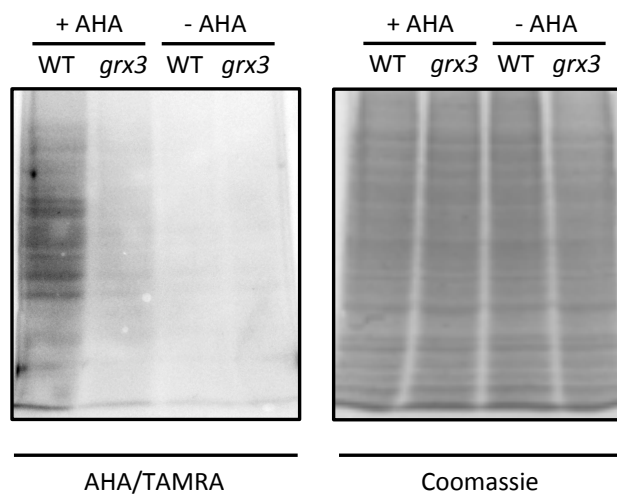
A



B



C



D

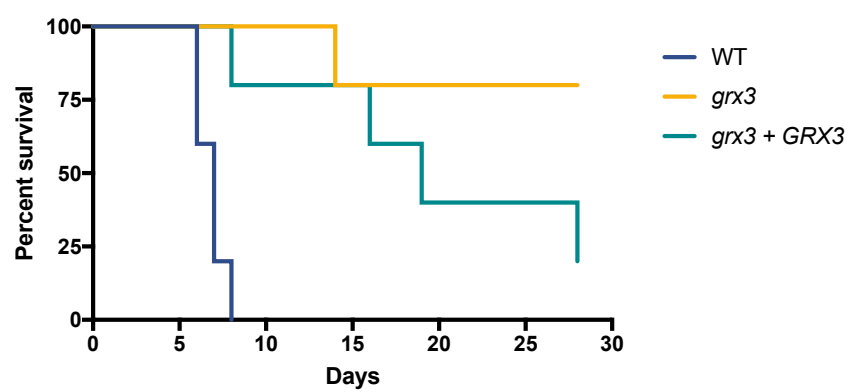


Figure 4.6. Grx3 plays roles in nitrosative stress response, DNA damage pathway, and translational efficiency. **A.** Sensitivity of WT and *grx3* to nitrosative stress. WT (HLY4494) and *grx3* (HLY4492) were serially diluted and spotted onto YPD plates containing 2.5mM or 5mM DPTA NONOate and grown at 30°C for 2-3 days. **B.** Sensitivity of WT and *grx3* to genotoxic stress. WT (HLY4494) and *grx3* (HLY4492) were serially diluted and spotted onto plates containing 30mM HU or 0.03% MMS and grown at 30°C for 2-3 days. **C.** Translational efficiency of WT and *grx3*. WT (HLY4494) and *grx3* (HLY4492) cells were methionine starved overnight then treated with either 35μM AHA or methionine. Cells were grown for 1 hr at 30°C then collected, treated, and run on a polyacrylamide gel. Newly synthesized proteins labeled with AHA were detected by TAMRA fluorescence. The gel was subsequently stained with coomassie for total protein detection. **D.** Survival of mice infected with the indicated *C. albicans* strains. Mice were injected in the lateral tail vein with WT, *grx3*, and *grx3* cells complemented with *pTDH3-GRX3*.

and Ddr48 (Table 4.1), which have been shown to regulate Fe-S cluster-dependent DNA damage responses [156-159]. To assess the role of Grx3 in genomic maintenance and the DNA damage response, we spotted wild-type and *grx3* cells onto YPD plates containing the genotoxic stresses hydroxyurea (HU) or methyl methanesulfonate (MMS) and assayed for sensitivity (Figure 4.6B). *grx3* was hypersensitive to both compounds, demonstrating that Grx3 contributes to the maintenance of genome stability in response to genotoxic stress, likely through its role in Fe-S cluster trafficking (Figure 4.6B).

Our mass spectrometry dataset was highly enriched for various translational processes including initiation, elongation, and amino acid biosynthesis (Figure 4.5, Table 4.1). In order to assess the role of Grx3 in protein biosynthesis and translation, we utilized a Click chemistry approach to label *in vivo* protein synthesis using the methionine surrogate L-azidohomoalanine (AHA) and its reactive fluorescent alkyne, tetramethylrhodamine (TAMRA) (Figure 4.6C). Cells were starved of methionine overnight then treated with AHA to label nascent protein synthesis. Control samples were treated only with methionine (-AHA). After 1hr treatment with AHA, wild-type cells showed much higher levels of protein translation relative to the *grx3* mutant (Figure 4.6C, left panel). Indeed, the *grx3* mutant fluorescent signal was only slightly higher than background when compared to the - AHA samples (Figure 4.6C, left panel). Verification by coomassie staining of the same imaged gel indicated equal protein loading (Figure 4.5C, right panel). Therefore, Grx3 contributes to translational efficiency *in vivo*.

Grx3 is required for virulence in a murine model of systemic infection

As iron acquisition from the host environment is essential for *C. albicans* and other host-dwelling organisms' ability to survive, we examined the effects of deleting *GRX3* on *C. albicans* virulence in a murine systemic infection model. Mice infected with wild-type cells all died by day 8, whereas most of mice infected with *grx3* mutant cells were still alive by day 28 (Figure 4.6D). Therefore, the *grx3* mutant showed an extremely marked decrease in virulence compared to the wild-type strain. A rescued *grx3* mutant carrying a single copy of *GRX3* under its own promoter was unable to fully complement the *grx3* phenotype as the survival rate for mice infected with the strain was better than that of wild-type, but worse than that of *grx3* mutant (Figure 4.6D). We have constructed another *GRX3* complemented strain with *GRX3* under a strong constitutive promoter for further testing in mice. Our preliminary data suggest that Grx3 contributes to *C. albicans* pathogenicity in the murine host.

DISCUSSION

Here we have shown that the *C. albicans* cytosolic monothiol glutaredoxin Grx3 is essential for the regulation of not only the iron homeostasis circuit, but also the regulation of many key processes dependent on Fe-S clusters for function. We compared four different *C. albicans* glutaredoxins and identified Grx3 an iron sensor and regulator. Using molecular genetic approaches we determined that Grx3 regulates the iron homeostasis circuit in at least three ways: (1) through regulation of Sfu1 DNA occupancy, (2) through the regulation of Sef1 localization, and (3) through the regulation

of Hap43 activity. Our genetic analyses hinted at a broader role of Grx3 in the regulation of other processes, the extent of which was demonstrated by our proteomic approach. Purification of Grx3 coupled with mass spectrometry enriched for interacting proteins involved in a variety of important processes including Fe-S cluster biogenesis and trafficking, oxidative stress response, metabolism, DNA maintenance and repair, and protein translation (Figure 4.7). We validated the essentiality of Grx3 in the proper function of several of these processes including redox homeostasis and stress response, response to genotoxic stress, and translational efficiency. The dependence of these processes on Fe-S cofactors and the known function of glutaredoxins in Fe-S cluster biogenesis and trafficking lead us to conclude that *C. albicans* Grx3 is a global iron sensor and Fe-S delivery protein necessary for the regulation of a multitude of Fe-S cluster-dependent pathways and processes occurring within the cell.

We determined that Grx3 functions as an iron sensor that contributes to the regulation of the *C. albicans* iron homeostasis circuit, as loss of *grx3* results in the regulatory circuit's insensitivity to iron level. We've shown that Grx3 regulates several aspects of the iron regulatory circuit, including Sfu1 activity, Sef1 localization, and Hap43 activity. This is consistent with work in other fungi that have linked cytosolic glutaredoxins to the regulation of their respective iron regulons [67, 160]. For example, we observed that Grx3 interacts with Sfu1 to control its occupancy at the *SEF1* promoter. In *S. pombe* Grx4 also controls the promoter association of the Sfu1 homolog Fep1. We show that Grx3 regulates Hap43 activity, although we have not addressed how Grx3 regulates Hap43 activity. In *S. pombe*, Grx4 is responsible for the cytosolic

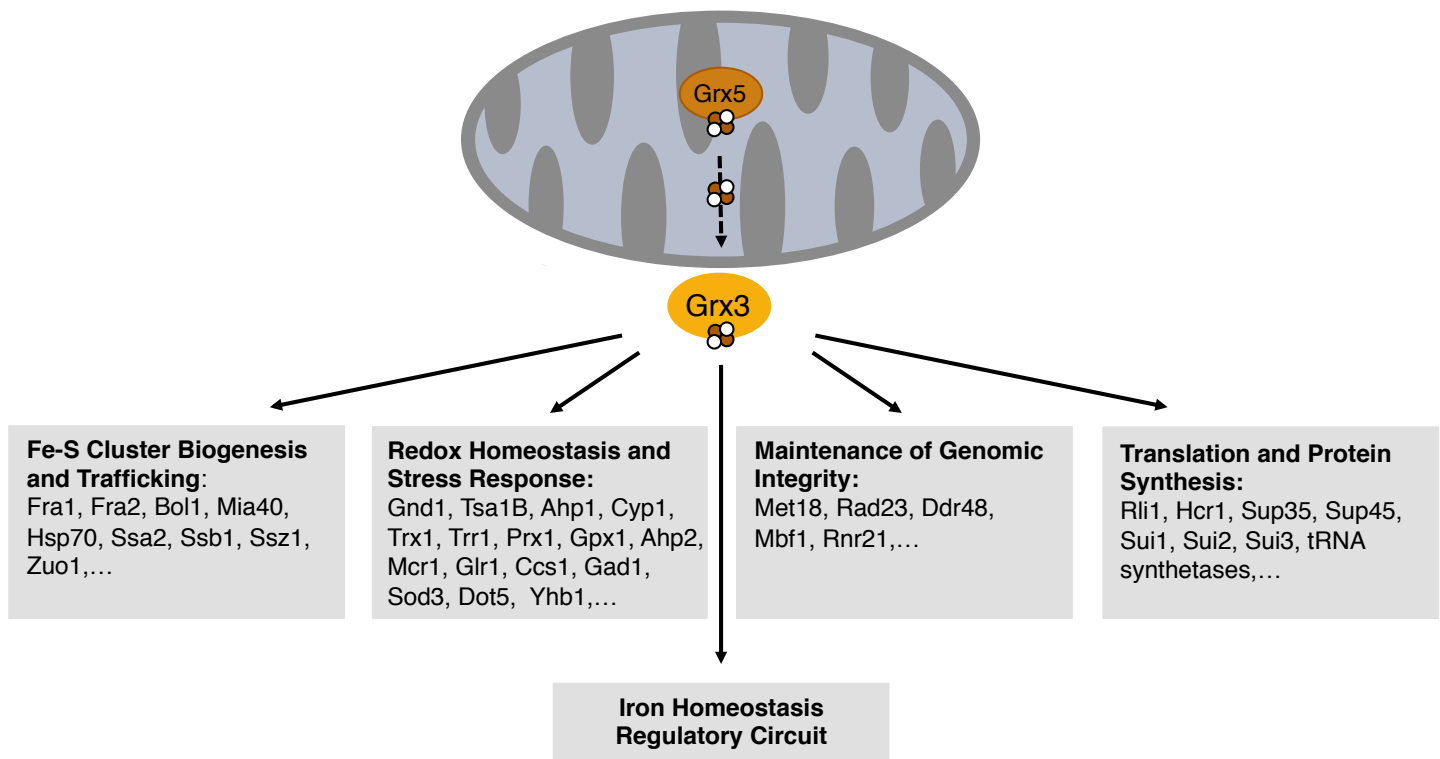


Figure 4.7. Grx3 is a global iron sensor and Fe-S carrier critical for the function of multiple Fe-S protein-dependent pathways. The Fe-S cluster-carrying protein Grx3 is an intracellular iron sensor and Fe-S trafficker that contributes to the function of a multitude of proteins involved in a wide range of biological processes, including the iron homeostasis circuit, redox homeostasis and response to oxidative and nitrosative stresses, maintenance of genomic integrity, metabolism and biosynthetic processes, and translation and protein synthesis.

sequestration of the Hap43 homolog Php4 under iron sufficiency. It is possible a similar mechanism exists in *C. albicans*, although this is yet to be determined experimentally.

By mass spectrometry we observed Grx3 interacting with stress response proteins essential for redox homeostasis of the cell, as well as several known Fe-S cluster-binding proteins including the BolA-like proteins encoded by *orf19.7489.3* (Bol2 in *S. cerevisiae* and Fra2 in *S. pombe*), *orf19.5016* (Bol1 in *S. cerevisiae* and Uvi31 in *S. pombe*), and *orf19.2095* (Fra1 in *S. cerevisiae* and *S. pombe*), which are well characterized proteins involved in Fe-S cluster biogenesis and trafficking (Figure 4.5, Table 4.1) [52, 61, 136, 139]. Fra/BolA proteins have also been shown to function in the regulation of iron homeostasis pathways in other fungi, such as *S. cerevisiae* and *S. pombe*, and it is possible that they function in a similar capacity in *C. albicans*, although that has yet to be seen [52, 136]. *S. cerevisiae* is unique in that it utilizes a mechanism independent from the conserved homeostasis regulons that exist in *S. pombe* and other pathogenic fungi, instead depending on the actions of the transcriptional regulators Aft1 and Aft2. In iron replete conditions an ScGrx3/4-Fra1/2 complex binds and sequesters Aft1/2 in the cytosol, preventing its nuclear localization and blocking Aft1/2-mediated induction of iron uptake genes [67]. Orf19.2095, a homolog of *S. cerevisiae* Fra1, was one of the top hits observed in our mass spectrometry data (Table 4.1). *C. albicans* also possesses an Aft homolog, Aft2, which has been shown to contribute to the regulation of iron response genes [161]. Therefore it is possible that *C. albicans* has retained a Grx3-Fra1-mediated mechanism to regulate iron response genes via Aft2. In fact, the pathogenic *Candida* species *C. glabrata*, a closer relative to *S. cerevisiae*, displays a

hybrid system involving both Aft1 and Sef1 factors in the regulation of its iron network [58].

Our mass spectrometry data also revealed Grx3 interacting proteins involved in DNA damage response and purine biosynthesis. The processes of DNA damage repair and DNA maintenance have been widely reported to depend on iron availability and Fe-S cluster cofactors [127, 152, 158]. Our dataset included proteins such as Met18 (*S. cerevisiae* Mms19), a component of cytosolic Fe-S protein assembly (CIA) that also plays roles in DNA replication and repair [156, 157], Rad23, a subunit of nuclear excision repair complex NEF2 in *S. cerevisiae* [162], Ddr48, a DNA damage response protein with a role in flocculation [163, 164], and Rnr21 (*S. cerevisiae* Rnr2), a subunit of ribonucleotide reductase (RNR), an iron-dependent enzyme involved in deoxyribonucleotide biosynthesis shown to be regulated by cellular iron level [165] (Table 4.1). The finding of many Grx3-interacting proteins playing pivotal roles in multiple DNA synthesis and repair pathways is consistent with increased sensitivity to genotoxic stress in the *grx3* mutant.

Our results also yielded enrichment for pathways including glycolysis and mannose metabolism, the pentose phosphate pathway, and flavin biosynthesis (Figure 4.5). It is known that under iron limitation flavin production is upregulated in bacteria, fungi including *C. albicans*, and plants [166-168]. In *Candida famata*, this process is regulated by Sef1 [169]. It is proposed that flavin production and excretion under iron limitation is a strategy of iron acquisition through a reduction of insoluble environmental

Fe³⁺ to soluble Fe²⁺ which is subsequently uptaken by the cell via iron transporters [170]. Therefore, it is expected to find the iron sensor Grx3 might regulate this process.

We observed a high number of ribosomal proteins, translation initiation factors, amino acid biosynthesis proteins, and tRNA synthetases in our Grx3 interaction data (Figure 4.5, Table 4.1), processes also known to be Fe-S cluster dependent. The RNase L inhibitor protein Rli1 is an Fe-S cluster protein essential for ribosome biogenesis, translation initiation, and translation termination [171, 172]. It has been shown that Rli1 is regulated by the Sfu1/Sef1/Hap43 iron regulatory circuit in *C. albicans* [51]. Rli1 interacts with the eIF3 component Hcr1 and the termination factors Sup35 and Sup45, all of which were identified to interact with Grx3 in our mass spectrometry data (Table 4.1) [173]. Further, Fe-S clusters have been implicated in regulation of tRNA modifications such as thiolation, and several bacterial tRNA synthetases have been shown to bind Fe-S clusters [172, 174-176].

These data, described here in brief, reveal the multitude of cellular processes regulated by the iron sensor and Fe-S shuttle protein Grx3. This is the first proteomic approach to identify Grx3 targets. Most of the proteins are shown for the first time to interact with Grx3. Conversely, many of the Grx3 associated proteins are previously shown to contain or depend on Fe-S clusters, further validated our findings. Our work suggests a wide role for Grx3 in overall Fe-S regulation of an assortment of biological processes and pathways. Due to the ubiquitous nature of glutaredoxins across all kingdoms of life, we believe it likely that the functional relationships between monothiol

glutathione reductases and the biological processes described in this study should show wide conservation throughout the living world.

CHAPTER 5

Summary and Conclusions

The opportunistic pathogen *C. albicans* is a fascinating organism to study. In addition to being clinically relevant, its extreme flexibility in host niche colonization, facilitated by its morphological and genetic plasticity, means it has evolved endless mechanisms to respond to the disparate host destinations it finds itself. Whether by epigenetic regulation of cell fate or regulation through a cofactor-binding and trafficking protein, *C. albicans* is well suited to adjust to the vagaries of life in the host. In this dissertation we have addressed two fundamental questions about *C. albicans* biology by utilizing a powerful mass spectrometry based approach.

The epigenetic transition between the white and opaque phases represents one mechanism by which *C. albicans* can alter its biology to best suit its environment. As presented in Chapter 3, we find that the master regulator of the opaque phase Wor1 induces and maintains the opaque state through inhibition of the general co-repressor Tup1. This interaction depends on their association at the level of promoter chromatin, and *WOR1* expression is sensitive to alterations in Wor1 or Tup1 level at the *WOR1* promoter. Changes in promoter occupancy of either Wor1 or Tup1 results in changes to *WOR1* expression and the transition between phases. Additional regulators such as Wor2, Wor3, Czf1, Efg1, and others all contribute to the regulation of *WOR1* expression. As Tup1 does not bind DNA directly, it is targeted to promoter regions through its

interaction with transcription factors. It is possible that the other white-opaque regulators (or those as yet undiscovered) contribute to the regulation of white-opaque switching through their interaction with and targeting of Tup1 to the *WOR1* promoter.

External cues such as carbon source, CO₂ level, hypoxia, and temperature all contribute to white-opaque switching. In Chapter 3, we examine two such environmental conditions, carbon source and temperature, and propose a molecular model for how they contribute to white-opaque switching. We determine that non-glycolytic carbon sources and growth at the 37°C both contribute to Wor1 and Tup1 occupancy at the *WOR1* promoter and consequently, regulate *WOR1* expression. We can postulate that other environmental conditions that regulate white-opaque switching may regulate Wor1 and Tup1 occupancy at the *WOR1* promoter as well. It is also possible these conditions may act on other white-opaque transcription factors, especially Efg1, which was observed in our mass spectrometry data and is known to physically interact with Tup1. These transcription factors in turn may alter Tup1 occupancy (and possibly activity) to regulate *WOR1* expression and cell fate. Therefore, the local chromatin environment at the level of the *WOR1* promoter is a critical determinant of opaque cell fate. The new paradigm we describe here can serve as a model for how other environmental conditions can regulate the *WOR1* promoter and white-opaque switching. Future work about how these signals are integrated to regulate opaque cell fate should provide ample room for expansion of our model.

Bioavailable iron level is another critical signal that *C. albicans* senses and responds to, both due to the requirement for iron is a myriad of essential biological

processes and the generation of toxic reactive oxygen species in the case of iron excess. In Chapter 4, we discuss the Grx3-mediated mechanism by which *C. albicans* can sense and responds to iron status. Monothiol glutaredoxins like Grx3 serve as iron sensors by binding Fe-S clusters and trafficking them to client proteins and modulating client protein function in a variety of ways. We find that Grx3 is essential for sensing iron status and regulating the iron homeostasis regulon. A conserved circuit present in a broad range of fungal species from the fission yeast *S. pombe* to the pathogenic *C. neoformans*, the iron homeostasis circuit ensures survival in times of iron sufficiency and iron limitation. At its simplest, the circuit contains the iron uptake repressor CaSfu1 (SpFep1) and the iron utilization repressor CaHap43 (SpPhp4), which display an antagonistic relationship to each other and repress the other's expression. We show that Grx3, as a conserved iron sensor, acts on both of these factors to regulate their activity in response to iron level. As an inhabitant of many diverse and dynamic host niches, *C. albicans* must be able to adapt to an extreme range of iron levels. Perhaps this unique requirement for extreme flexibility is why *C. albicans* has evolved and incorporated a new component into the conserved iron homeostasis regulon in the form of Sef1. In agreement with its known role as an iron sensor, we show that Grx3 also regulates Sef1 at the level of its localization.

Beyond its ability to regulate the iron homeostasis circuit, Grx3 is also critical for the regulation of a diverse array of biological processes, including Fe-S biogenesis, redox homeostasis, metabolism, DNA synthesis and repair, and protein translation. Many of these processes are known to be regulated by iron, and many of the Grx3-

associated proteins we identified have been shown to bind Fe-S clusters or be dependent on their presence for activity. Therefore Grx3 is a global iron sensor and trafficker that is crucial for the overall wellbeing and adequate function of the cell. As Fe-S cluster biogenesis occurs in the mitochondria and is dependent on mitochondrial function, it becomes clear that the mitochondria are more than just the powerhouse of the cell. Instead, by regulating processes such as Fe-S cluster biogenesis, the mitochondria becomes an integral signaling component of the cell.

Future work can dissect exactly how the mitochondria, via processes such as Fe-S cluster biogenesis and trafficking, or metabolic shifts in the case of white-opaque switching, can serve as a signal intermediate to convert environmental cues and conditions into the regulation of gene expression, cellular activity, and cell fate.

REFERENCES

1. Pfaller MA, Diekema DJ. Epidemiology of invasive candidiasis: a persistent public health problem. *Clin Microbiol Rev.* 2007;20:133-63.
2. Vincent J-L, Rello J, Marshal J, Silva E, Anzueto A, Martin CD. International study of the prevalence and outcomes of infection in intensive care units. *JAMA.* 2009;302:2323-9.
3. Rikkerink EHA, Magee BB, Magee PT. Opaque-White Phenotype Transition: a Programmed Morphological Transition in *Candida albicans*. *Journal of bacteriology.* 1988;170(2):895-9.
4. Tuch BB, Mitrovich QM, Homann OR, Hernday AD, Monighetti CK, De La Vega FM, et al. The transcriptomes of two heritable cell types illuminate the circuit governing their differentiation. *PLoS genetics.* 2010;6(8):e1001070. doi: 10.1371/journal.pgen.1001070. PubMed PMID: 20808890; PubMed Central PMCID: PMC2924316.
5. Lan CY, Newport G, Murillo LA, Jones T, Scherer S, Davis RW, et al. Metabolic specialization associated with phenotypic switching in *Candida albicans*. *Proceedings of the National Academy of Sciences of the United States of America.* 2002;99:14907-12.
6. Kvaal CA, Lachke SA, Srikantha T, Daniels K, McCoy J, Soll DR. Misexpression of the Opaque-Phase-Specific Gene PEP1 (SAP1) in the White Phase of *Candida albicans* Confers Increased Virulence in a Mouse Model of Cutaneous Infection. *Infect Immun.* 1999;67(12):6652-62.
7. Lachke SA, Lockhart SR, Daniels KJ, Soll DR. Skin Facilitates *Candida albicans* Mating. *Infection and Immunity.* 2003;71(9):4970-6. doi: 10.1128/iai.71.9.4970-4976.2003.
8. Lohse MB, Johnson AD. Differential phagocytosis of white versus opaque *Candida albicans* by *Drosophila* and mouse phagocytes. *PloS one.* 2008;3(1):e1473. doi: 10.1371/journal.pone.0001473. PubMed PMID: 18213381; PubMed Central PMCID: PMC2198939.
9. Mallick EM, Bergeron AC, Jones SK, Jr., Newman ZR, Brothers KM, Creton R, et al. Phenotypic Plasticity Regulates *Candida albicans* Interactions and Virulence in the Vertebrate Host. *Front Microbiol.* 2016;7:780. doi: 10.3389/fmicb.2016.00780. PubMed PMID: 27303374; PubMed Central PMCID: PMCPMC4880793.
10. Huang G, Yi S, Sahni N, Daniels KJ, Srikantha T, Soll DR. N-Acetylglucosamine Induces White to Opaque Switching, a Mating Prerequisite in *Candida albicans*. *PLoS pathogens.* 2010;6(3):e1000806. doi: 10.1371/journal.ppat.1000806.
11. Huang G, Srikantha T, Sahni N, Yi S, Soll DR. CO₂ regulates white-to-opaque switching in *Candida albicans*. *Current biology : CB.* 2009;19(4):330-4.
12. Dumitru R, Navarathna DH, Semighini CP, Elowsky CG, Dumitru RV, Dignard D, et al. In vivo and in vitro anaerobic mating in *Candida albicans*. *Eukaryotic cell.* 2007;6(3):465-72. doi: 10.1128/EC.00316-06. PubMed PMID: 17259544; PubMed Central PMCID: PMCPMC1828919.

13. Ramirez-Zavala B, Reuss O, Park YN, Ohlsen K, Morschhauser J. Environmental induction of white-opaque switching in *Candida albicans*. PLoS pathogens. 2008;4(6):e1000089. doi: 10.1371/journal.ppat.1000089. PubMed PMID: 18551173; PubMed Central PMCID: PMCPMC2405950.
14. Morrow B, Anderson JB, Wilson J, Soll DR. Bidirectional stimulation of the white-opaque transition of *Candida albicans* by ultraviolet irradiation. J Gen Microbiol. 1989;135(5):1201-8.
15. Stevenson JS, Liu H. Regulation of white and opaque cell-type formation in *Candida albicans* by Rtt109 and Hst3. Molecular microbiology. 2011;81(4):1078-91. doi: 10.1111/j.1365-2958.2011.07754.x. PubMed PMID: 21749487; PubMed Central PMCID: PMCPMC4049571.
16. Stevenson JS, Liu H. Nucleosome Assembly Factors CAF-1 and HIR Modulate Epigenetic Switching Frequencies in an H3K56 Acetylation Associated Manner in *Candida albicans*. Eukaryotic cell. 2013;12(4):591-603.
17. Miller MG, Johnson AD. White-opaque switching in *Candida albicans* is controlled by mating-type locus homeodomain proteins and allows efficient mating. Cell. 2002;110(3):293-302.
18. Lockhart SR, Pujol C, Daniels KJ, Miller MG, Johnson AD, Pfaller M, et al. In *Candida albicans*, White-Opaque Switchers Are Homozygous for Mating Type. Genetics. 2002;162(2):737-45.
19. Xie J, Tao L, Nobile CJ, Tong Y, Guan G, Sun Y, et al. White-opaque switching in natural *MTLa/a* isolates of *Candida albicans*: evolutionary implications for roles in host adaptation, pathogenesis, and sex. PLoS biology. 2013;11(3):e1001525. doi: 10.1371/journal.pbio.1001525. PubMed PMID: 23555196; PubMed Central PMCID: PMC3608550.
20. Pande K, Chen C, Noble SM. Passage through the mammalian gut triggers a phenotypic switch that promotes *Candida albicans* commensalism. Nature genetics. 2013;45(9):1088-91. doi: 10.1038/ng.2710. PubMed PMID: 23892606; PubMed Central PMCID: PMC3758371.
21. Huang G, Wang H, Chou S, Nie X, Chen J, Liu H. Bistable expression of *WOR1*, a master regulator of white-opaque switching in *Candida albicans*. Proceedings of the National Academy of Sciences of the United States of America. 2006;103(34):12813-8. doi: 10.1073/pnas.0605270103. PubMed PMID: 16905649; PubMed Central PMCID: PMC1540355.
22. Srikantha T, Borneman AR, Daniels KJ, Pujol C, Wu W, Seringhaus MR, et al. *TOS9* regulates white-opaque switching in *Candida albicans*. Eukaryotic cell. 2006;5(10):1674-87. doi: 10.1128/EC.00252-06. PubMed PMID: 16950924; PubMed Central PMCID: PMCPMC1595353.
23. Zordan RE, Galgoczy DJ, Johnson AD. Epigenetic properties of white-opaque switching in *Candida albicans* are based on a self-sustaining transcriptional feedback loop. Proceedings of the National Academy of Sciences of the United States of America. 2006;103(34):12807-12. doi: 10.1073/pnas.0605138103. PubMed PMID: 16899543; PubMed Central PMCID: PMC1535343.

24. Guan Z, Liu H. Overlapping Functions between *SWR1* Deletion and H3K56 Acetylation in *Candida albicans*. Eukaryotic cell. 2015;14(6):578-87. doi: 10.1128/EC.00002-15. PubMed PMID: 25862154; PubMed Central PMCID: PMC4452568.
25. Hnisz D, Schwarzmuller T, Kuchler K. Transcriptional loops meet chromatin: a dual-layer network controls white-opaque switching in *Candida albicans*. Molecular microbiology. 2009;74(1):1-15. doi: 10.1111/j.1365-2958.2009.06772.x. PubMed PMID: 19555456; PubMed Central PMCID: PMCPMC2764112.
26. Xie J, Jenull S, Tscherner M, Kuchler K. The Paralogous Histone Deacetylases Rpd3 and Rpd31 Play Opposing Roles in Regulating the White-Opaque Switch in the Fungal Pathogen *Candida albicans*. MBio. 2016;7(6). doi: 10.1128/mBio.01807-16. PubMed PMID: 27935838; PubMed Central PMCID: PMCPMC5111407.
27. Guan Z, Liu H. The *WOR1* 5' untranslated region regulates white-opaque switching in *Candida albicans* by reducing translational efficiency. Molecular microbiology. 2015;97(1):125-38. doi: 10.1111/mmi.13014. PubMed PMID: 25831958.
28. Hernday AD, Lohse MB, Fordyce PM, Nobile CJ, DeRisi JL, Johnson AD. Structure of the transcriptional network controlling white-opaque switching in *Candida albicans*. Molecular microbiology. 2013;90(1):22-35. doi: 10.1111/mmi.12329. PubMed PMID: 23855748; PubMed Central PMCID: PMC3888361.
29. Lohse MB, Rosenberg OS, Cox JS, Stroud RM, Finer-Moore JS, Johnson AD. Structure of a new DNA-binding domain which regulates pathogenesis in a wide variety of fungi. Proceedings of the National Academy of Sciences of the United States of America. 2014;111(29):10404-10. doi: 10.1073/pnas.1410110111. PubMed PMID: 24994900; PubMed Central PMCID: PMC4115540.
30. Vences MD, Kumamoto CA. The morphogenetic regulator Czf1p is a DNA-binding protein that regulates white opaque switching in *Candida albicans*. Microbiology. 2007;153(Pt 9):2877-84. doi: 10.1099/mic.0.2007/005983-0. PubMed PMID: 17768232.
31. Zordan RE, Miller MG, Galgoczy DJ, Tuch BB, Johnson AD. Interlocking transcriptional feedback loops control white-opaque switching in *Candida albicans*. PLoS biology. 2007;5(10):e256. doi: 10.1371/journal.pbio.0050256. PubMed PMID: 17880264; PubMed Central PMCID: PMC1976629.
32. Cain CW, Lohse MB, Homann OR, Sil A, Johnson AD. A conserved transcriptional regulator governs fungal morphology in widely diverged species. Genetics. 2012;190(2):511-21. doi: 10.1534/genetics.111.134080. PubMed PMID: 22095082; PubMed Central PMCID: PMC3276625.
33. Lohse MB, Zordan RE, Cain CW, Johnson AD. Distinct class of DNA-binding domains is exemplified by a master regulator of phenotypic switching in *Candida albicans*. Proceedings of the National Academy of Sciences of the United States of America. 2010;107(32):14105-10. doi: 10.1073/pnas.1005911107. PubMed PMID: 20660774; PubMed Central PMCID: PMC2922561.
34. Michielse CB, van Wijk R, Reijnen L, Manders EM, Boas S, Olivain C, et al. The nuclear protein Sge1 of *Fusarium oxysporum* is required for parasitic growth. PLoS pathogens. 2009;5(10):e1000637. doi: 10.1371/journal.ppat.1000637. PubMed PMID: 19851506; PubMed Central PMCID: PMC2762075.

35. Nguyen VQ, Sil A. Temperature-induced switch to the pathogenic yeast form of *Histoplasma capsulatum* requires Ryp1, a conserved transcriptional regulator. *Proceedings of the National Academy of Sciences of the United States of America*. 2008;105(12):4880-5. doi: 10.1073/pnas.0710448105. PubMed PMID: 18339808; PubMed Central PMCID: PMC2290814.
36. Zhang S, Zhang T, Yan M, Ding J, Chen J. Crystal structure of the WOPR-DNA complex and implications for Wor1 function in white-opaque switching of *Candida albicans*. *Cell Res*. 2014;24:1108-20.
37. Almeida RS, Wilson D, Hube B. *Candida albicans* iron acquisition within the host. *FEMS Yeast Res*. 2009;9(7):1000-12. doi: 10.1111/j.1567-1364.2009.00570.x. PubMed PMID: 19788558.
38. Noble SM. *Candida albicans* specializations for iron homeostasis: from commensalism to virulence. *Curr Opin Microbiol*. 2013;16(6):708-15. doi: 10.1016/j.mib.2013.09.006. PubMed PMID: 24121029; PubMed Central PMCID: PMCPMC3855070.
39. Pierre JL, Crichton RR. Chemistry for an essential biological process: the reduction of ferric iron. *Biometals*. 2002;15(4):341-6.
40. Hennigar SR, McClung JP. Nutritional immunity: starving pathogens of trace minerals. *American Journal of Lifestyle Medicine*. 2016;10(3):170-3. doi: 10.1177/1559827616629117.
41. Bullen JJ, Rogers HJ, Spalding PB, Ward CG. Natural resistance, iron and infection: a challenge for clinical medicine. *J Med Microbiol*. 2006;55(Pt 3):251-8. doi: 10.1099/jmm.0.46386-0. PubMed PMID: 16476787.
42. Evans DM, Frazer IH, Martin NG. Genetic and environmental causes of variation in basal levels of blood cells. *Twin Research*. 2012;2(04):250-7. doi: 10.1375/twin.2.4.250.
43. Bali PK, Aisen P. Receptor-induced switch in site-site cooperativity during iron release by transferrin. *Biochemistry*. 1992;31(16):3963-7.
44. Friedman DB, Stauff DL, Pishchany G, Whitwell CW, Torres VJ, Skaar EP. *Staphylococcus aureus* redirects central metabolism to increase iron availability. *PLoS Pathog*. 2006;2(8):e87. doi: 10.1371/journal.ppat.0020087. PubMed PMID: 16933993; PubMed Central PMCID: PMCPMC1557832.
45. Ziegler L, Terzulli A, Gaur R, McCarthy R, Kosman DJ. Functional characterization of the ferroxidase, permease high-affinity iron transport complex from *Candida albicans*. *Mol Microbiol*. 2011;81(2):473-85. doi: 10.1111/j.1365-2958.2011.07704.x. PubMed PMID: 21645130; PubMed Central PMCID: PMCPMC3133879.
46. Harrison PM. The structure and function of ferritin. *Biochem Educ*. 1986;14(4):154-62.
47. Almeida RS, Brunke S, Albrecht A, Thewes S, Laue M, Edwards JE, et al. The hyphal-associated adhesin and invasin Als3 of *Candida albicans* mediates iron acquisition from host ferritin. *PLoS Pathog*. 2008;4(11):e1000217. doi: 10.1371/journal.ppat.1000217. PubMed PMID: 19023418; PubMed Central PMCID: PMCPMC2581891.

48. Gobin J, Horwitz MA. Exochelins of *Mycobacterium tuberculosis* remove iron from human iron-binding proteins and donate iron to mycobactins in the *M. tuberculosis* cell wall. *J Exp Med*. 1996;183(4):1527-32.
49. Haas H. Molecular genetics of fungal siderophore biosynthesis and uptake: the role of siderophores in iron uptake and storage. *Appl Microbiol Biotechnol*. 2003;62(4):316-30. doi: 10.1007/s00253-003-1335-2. PubMed PMID: 12759789.
50. Odds FC. *Candida* and candidosis: A Review and Bibliography: Bailliere Tindall, London; 1988.
51. Chen C, Pande K, French SD, Tuch BB, Noble SM. An iron homeostasis regulatory circuit with reciprocal roles in *Candida albicans* commensalism and pathogenesis. *Cell Host Microbe*. 2011;10(2):118-35. doi: 10.1016/j.chom.2011.07.005. PubMed PMID: 21843869; PubMed Central PMCID: PMC3165008.
52. Encinar del Dedo J, Gabrielli N, Carmona M, Ayte J, Hidalgo E. A cascade of iron-containing proteins governs the genetic iron starvation response to promote iron uptake and inhibit iron storage in fission yeast. *PLoS Genet*. 2015;11(3):e1005106. doi: 10.1371/journal.pgen.1005106. PubMed PMID: 25806539; PubMed Central PMCID: PMC4373815.
53. Jung WH, Kronstad JW. Iron influences the abundance of the iron regulatory protein Cir1 in the fungal pathogen *Cryptococcus neoformans*. *FEBS Lett*. 2011;585(20):3342-7. doi: 10.1016/j.febslet.2011.09.025. PubMed PMID: 21963719; PubMed Central PMCID: PMC3200532.
54. Jung WH, Saikia S, Hu G, Wang J, Fung CK, D'Souza C, et al. HapX positively and negatively regulates the transcriptional response to iron deprivation in *Cryptococcus neoformans*. *PLoS Pathog*. 2010;6(11):e1001209. doi: 10.1371/journal.ppat.1001209. PubMed PMID: 21124817; PubMed Central PMCID: PMC2991262.
55. Chao LY, Marletta MA, Rine J. Sre1, an Iron-Modulated GATA DNA-Binding Protein of Iron-Uptake Genes in the Fungal Pathogen *Histoplasma capsulatum*. *Biochemistry*. 2008;47(27):7274-83.
56. Hwang LH, Seth E, Gilmore SA, Sil A. *SRE1* regulates iron-dependent and -independent pathways in the fungal pathogen *Histoplasma capsulatum*. *Eukaryot Cell*. 2012;11(1):16-25. doi: 10.1128/EC.05274-11. PubMed PMID: 22117028; PubMed Central PMCID: PMC3255934.
57. Chen C, Noble SM. Post-transcriptional regulation of the Sef1 transcription factor controls the virulence of *Candida albicans* in its mammalian host. *PLoS Pathog*. 2012;8(11):e1002956. doi: 10.1371/journal.ppat.1002956. PubMed PMID: 23133381; PubMed Central PMCID: PMC3486892.
58. Gerwien F, Safyan A, Wisgott S, Hille F, Kaemmer P, Linde J, et al. A Novel Hybrid Iron Regulation Network Combines Features from Pathogenic and Nonpathogenic Yeasts. *MBio*. 2016;7(5). doi: 10.1128/mBio.01782-16. PubMed PMID: 27795405; PubMed Central PMCID: PMC5082906.
59. Couturier J, Przybyla-Toscano J, Roret T, Didierjean C, Rouhier N. The roles of glutaredoxins ligating Fe-S clusters: Sensing, transfer or repair functions? *Biochim Biophys Acta*. 2015;1853(6):1513-27. doi: 10.1016/j.bbamcr.2014.09.018. PubMed PMID: 25264274.

60. Herrero E, de la Torre-Ruiz MA. Monothiol glutaredoxins: a common domain for multiple functions. *Cell Mol Life Sci.* 2007;64(12):1518-30. doi: 10.1007/s00018-007-6554-8. PubMed PMID: 17415523.
61. Li H, Outten CE. Monothiol CGFS glutaredoxins and BolA-like proteins: [2Fe-2S] binding partners in iron homeostasis. *Biochemistry.* 2012;51(22):4377-89. doi: 10.1021/bi300393z. PubMed PMID: 22583368; PubMed Central PMCID: PMC3448021.
62. Shenton D, Perrone G, Quinn KA, Dawes IW, Grant CM. Regulation of protein S-thiolation by glutaredoxin 5 in the yeast *Saccharomyces cerevisiae*. *J Biol Chem.* 2002;277(19):16853-9. doi: 10.1074/jbc.M200559200. PubMed PMID: 11882660.
63. Shenton D, Grant CM. Protein S-thiolation targets glycolysis and protein synthesis in response to oxidative stress in the yeast *Saccharomyces cerevisiae*. *Biochem J.* 2003;374(Pt 2):513-9.
64. Zaffagnini M, Michelet L, Massot V, Trost P, Lemaire SD. Biochemical characterization of glutaredoxins from *Chlamydomonas reinhardtii* reveals the unique properties of a chloroplastic CGFS-type glutaredoxin. *J Biol Chem.* 2008;283(14):8868-76. doi: 10.1074/jbc.M709567200. PubMed PMID: 18216016.
65. Braymer JJ, Lill R. Iron-sulfur cluster biogenesis and trafficking in mitochondria. *J Biol Chem.* 2017;292(31):12754-63. doi: 10.1074/jbc.R117.787101. PubMed PMID: 28615445; PubMed Central PMCID: PMC5546016.
66. Sheftel A, Stehling O, Lill R. Iron-sulfur proteins in health and disease. *Trends Endocrinol Metab.* 2010;21(5):302-14. doi: 10.1016/j.tem.2009.12.006. PubMed PMID: 20060739.
67. Martinez-Pastor MT, Perea-Garcia A, Puig S. Mechanisms of iron sensing and regulation in the yeast *Saccharomyces cerevisiae*. *World J Microbiol Biotechnol.* 2017;33(4):75. doi: 10.1007/s11274-017-2215-8. PubMed PMID: 28315258.
68. Mercier A, Pelletier B, Labbe S. A transcription factor cascade involving Fep1 and the CCAAT-binding factor Php4 regulates gene expression in response to iron deficiency in the fission yeast *Schizosaccharomyces pombe*. *Eukaryot Cell.* 2006;5(11):1866-81. doi: 10.1128/EC.00199-06. PubMed PMID: 16963626; PubMed Central PMCID: PMC1694796.
69. Jbel M, Mercier A, Labbe S. Grx4 monothiol glutaredoxin is required for iron limitation-dependent inhibition of Fep1. *Eukaryot Cell.* 2011;10(5):629-45. doi: 10.1128/EC.00015-11. PubMed PMID: 21421748; PubMed Central PMCID: PMC3127657.
70. Mercier A, Labbe S. Both Php4 function and subcellular localization are regulated by iron via a multistep mechanism involving the glutaredoxin Grx4 and the exportin Crm1. *J Biol Chem.* 2009;284(30):20249-62. doi: 10.1074/jbc.M109.009563. PubMed PMID: 19502236; PubMed Central PMCID: PMC2740451.
71. Tagwerker C, Flick K, Cui M, Guerrero C, Dou Y, Auer B, et al. A tandem affinity tag for two-step purification under fully denaturing conditions. *Molecular and Cellular Proteomics.* 2006;5(4):737-48. doi: 10.1074/.

72. Feng Q, Summers E, Guo B, Fink G. Ras Signaling Is Required for Serum-Induced Hyphal Differentiation in *Candida albicans*. *Journal of bacteriology*. 1999;181(20):6339-46.
73. Chen J, Chen J, Lane S, Liu H. A conserved mitogen-activated protein kinase pathway is required for mating in *Candida albicans*. *Molecular microbiology*. 2002;46(5):1335-44.
74. Noble SM, Johnson AD. Strains and strategies for large-scale gene deletion studies of the diploid human fungal pathogen *Candida albicans*. *Eukaryotic cell*. 2005;4(2):298-309. doi: 10.1128/EC.4.2.298-309.2005. PubMed PMID: 15701792; PubMed Central PMCID: PMC549318.
75. Hickman MA, Zeng G, Forche A, Hirakawa MP, Abbey D, Harrison BD, et al. The 'obligate diploid' *Candida albicans* forms mating-competent haploids. *Nature*. 2013;494(7435):55-9. doi: 10.1038/nature11865. PubMed PMID: 23364695; PubMed Central PMCID: PMC3583542.
76. Vyas VK, Barrasa MI, Fink GR. A *Candida albicans* CRISPR system permits genetic engineering of essential genes and gene families. *Sci Adv*. 2015;1(3):e1500248.
77. Fonzi WA, Irwin MY. Isogenic Strain Construction and Gene Mapping in *Candida albicans*. *Genetics*. 1993;134:717-28.
78. Roemer T, Jiang B, Davison J, Ketela T, Veillette K, Breton A, et al. Large-scale essential gene identification in *Candida albicans* and applications to antifungal drug discovery. *Molecular Microbiology*. 2003;50(1):167-81. doi: 10.1046/j.1365-2958.2003.03697.x.
79. Homann OR, Dea J, Noble SM, Johnson AD. A phenotypic profile of the *Candida albicans* regulatory network. *PLoS Genet*. 2009;5(12):e1000783. doi: 10.1371/journal.pgen.1000783. PubMed PMID: 20041210; PubMed Central PMCID: PMC2790342.
80. Alkafeef SS, Yu C, Huang L, Liu H. Wor1 establishes opaque cell fate through inhibition of the general co-repressor Tup1 in *Candida albicans*. *PLoS Genet*. 2018;14(1):e1007176. doi: 10.1371/journal.pgen.1007176. PubMed PMID: 29337983.
81. Yu C, Yang Y, Wang X, Guan S, Fang L, Liu F, et al. Characterization of Dynamic UbR Proteasome Subcomplexes by In vivo Cross-linking (X) Assisted Bimolecular Tandem Affinity Purification (XBAP) and Label-free Quantitation. *Mol Cell Proteomics*. 2016;15(7):2279-92. doi: 10.1074/.
82. Cox J, Hein MY, Luber CA, Paron I, Nagaraj N, Mann M. Accurate Proteome wide Label-free Quantification by Delayed Normalization and Maximal Peptide Ratio Extraction, Termed MaxLFQ. *Mol Cell Proteomics*. 2014;13(9):2513-26. doi: 10.1074/.
83. Schaab C, Geiger T, Stoehr G, Cox J, Mann M. Analysis of high accuracy, quantitative proteomics data in the MaxQB database. *Mol Cell Proteomics*. 2012;11(3):M111 014068. doi: 10.1074/mcp.M111.014068. PubMed PMID: 22301388; PubMed Central PMCID: PMC3316731.
84. Lu Y, Su C, Wang A, Liu H. Hyphal development in *Candida albicans* requires two temporally linked changes in promoter chromatin for initiation and maintenance. *PLoS biology*. 2011;9(7):e1001105. doi: 10.1371/journal.pbio.1001105. PubMed PMID: 21811397; PubMed Central PMCID: PMC3139633.

85. Sanchez AA, Johnston DA, Myers C, Edwards JE, Mitchell AP, Filler SG. Relationship between *Candida albicans* virulence during experimental hematogenously disseminated infection and endothelial cell damage in vitro. *Infection and Immunity*. 2003;72(1):598-601. doi: 10.1128/iai.72.1.598-601.2004.
86. Noble SM, Gianetti BA, Witchley JN. *Candida albicans* cell-type switching and functional plasticity in the mammalian host. *Nat Rev Microbiol*. 2017;15(2):96-108. doi: 10.1038/nrmicro.2016.157. PubMed PMID: 27867199.
87. Slutsky B, Staebell M, Anderson J, Risen L, Pfaller M, Soll DR. "White-opaque transition": a second high-frequency switching system in *Candida albicans*. *Journal of bacteriology*. 1987;169(1):189-97.
88. Su C, Lu Y, Liu H. N-acetylglucosamine sensing by a GCN5-related N-acetyltransferase induces transcription via chromatin histone acetylation in fungi. *Nat Commun*. 2016;7:12916. doi: 10.1038/ncomms12916. PubMed PMID: 27694804; PubMed Central PMCID: PMC5063960.
89. Lohse MB, Hernday AD, Fordyce PM, Noiman L, Sorrells TR, Hanson-Smith V, et al. Identification and characterization of a previously undescribed family of sequence-specific DNA-binding domains. *Proceedings of the National Academy of Sciences of the United States of America*. 2013;110(19):7660–5. doi: 10.1073/pnas.1221734110.
90. Buscarlet M, Stifani S. The 'Marx' of Groucho on development and disease. *Trends in cell biology*. 2007;17(7):353-61. doi: 10.1016/j.tcb.2007.07.002. PubMed PMID: 17643306.
91. Lee JE, Golz JF. Diverse roles of Groucho/Tup1 co-repressors in plant growth and development. *Plant Signal Behav*. 2012;7(1):86-92. doi: 10.4161/psb.7.1.18377. PubMed PMID: 22301974; PubMed Central PMCID: PMC3357377.
92. García-Sánchez S, Mavor AL, Russell CL, Argimon S, Dennison P, Enjalbert B, et al. Global roles of Ssn6 in Tup1- and Nrg1-dependent gene regulation in the fungal pathogen, *Candida albicans*. *Mol Biol Cell*. 2005;16:2913-25. doi: 10.1091/mbc.E05-.
93. Murad AMA, D'Enfert C, Gaillardin C, Tournu H, Tekaia F, Talibi D, et al. Transcript profiling in *Candida albicans* reveals new cellular functions for the transcriptional repressors CaTup1, CaMig1 and CaNrg1. *Molecular microbiology*. 2001;42(4):981-93.
94. Malave TM, Dent SY. Transcriptional repression by Tup1-Ssn6. *Biochem Cell Biol*. 2006;84(4):437-43. doi: 10.1139/o06-073. PubMed PMID: 16936817.
95. Smith RL, Johnson AD. Turning genes off by Ssn6-Tup1: a conserved system of transcriptional repression in eukaryotes. *Trends Biochem Sci*. 2000;25(7):325-30.
96. Santos MA, Keith G, Tuite MF. Non-standard translational events in *Candida albicans* mediated by an unusual seryl-tRNA with a 5'-CAG-3' (leucine) anticodon. *EMBO J*. 1993;12(2):607-16.
97. Keleher CA, Redd MJ, Schultz J, Carlson M, Johnson AD. Ssn6-Tup1 Is a General Repressor of Transcription in Yeast. *Cell*. 1992;68(4):709-19.
98. Varanasi US, Klis MK, Mikesell PB, Trumbly RJ. The Cyc8 (Ssn6)-Tup1 Corepressor Complex Is Composed of One Cyc8 and Four Tup1 Subunits. *Mol Cell Biol*. 1996;16(12):6707-14.

99. Kaneko A, Umeyama T, Utena-Abe Y, Yamagoe S, Niimi M, Uehara Y. Tcc1p, a novel protein containing the tetratricopeptide repeat motif, interacts with Tup1p to regulate morphological transition and virulence in *Candida albicans*. Eukaryotic cell. 2006;5(11):1894-905. doi: 10.1128/EC.00151-06. PubMed PMID: 16998076; PubMed Central PMCID: PMC1694794.
100. Dowell NL, Sperling AS, Mason MJ, Johnson RC. Chromatin-dependent binding of the *S. cerevisiae* HMGB protein Nhp6A affects nucleosome dynamics and transcription. Genes & development. 2010;24(18):2031-42. doi: 10.1101/gad.1948910. PubMed PMID: 20844014; PubMed Central PMCID: PMC2939365.
101. Zhang W, Zhang J, Zhang X, Xu C, Tu X. Solution structure of the Taf14 YEATS domain and its roles in cell growth of *Saccharomyces cerevisiae*. The Biochemical journal. 2011;436(1):83-90. doi: 10.1042/BJ20110004. PubMed PMID: 21355849.
102. Yan M, Nie X, Wang H, Gao N, Liu H, Chen J. SUMOylation of Wor1 by a novel SUMO E3 ligase controls cell fate in *Candida albicans*. Molecular microbiology. 2015;1-21. doi: 10.1111/mmi.13108.
103. Zhao R, Lockhart SR, Daniels K, Soll DR. Roles of *TUP1* in Switching, Phase Maintenance, and Phase-Specific Gene Expression in *Candida albicans*. Eukaryotic cell. 2002;1(3):353-65. doi: 10.1128/ec.1.3.353-365.2002.
104. Park YN, Morschhauser J. *Candida albicans* *MTLalpha tup1Δ* mutants can reversibly switch to mating-competent, filamentous growth forms. Molecular microbiology. 2005;58(5):1288-302. doi: 10.1111/j.1365-2958.2005.04898.x. PubMed PMID: 16313617.
105. Bergen MS, Voss E, Soll DR. Switching at the cellular level in the white-opaque transition of *Candida albicans*. J Gen Microbiol. 1990;136(10):1925-36.
106. Lohse MB, Johnson AD. Temporal anatomy of an epigenetic switch in cell programming: the white-opaque transition of *C. albicans*. Molecular microbiology. 2010;78(2):331-43. doi: 10.1111/j.1365-2958.2010.07331.x. PubMed PMID: 20735781; PubMed Central PMCID: PMC3057202.
107. Konopka JB. N-acetylglucosamine functions in cell signaling. Scientifica (Cairo). 2012;2012. doi: 10.6064/2012/489208. PubMed PMID: 23350039; PubMed Central PMCID: PMCPMC3551598.
108. Treitel MA, Carlson M. Repression by SSN6-TUP1 is directed by MIG1, a repressor activator protein. Proceedings of the National Academy of Sciences of the United States of America. 1995;92:3132-6.
109. Hernday AD, Lohse MB, Nobile CJ, Noiman L, Laksana CN, Johnson AD. Ssn6 Defines a New Level of Regulation of White-Opaque Switching in *Candida albicans* and Is Required For the Stochasticity of the Switch. MBio. 2016;7(1). doi: 10.1128/mBio.01565-15. PubMed PMID: 26814177.
110. Lohse MB, Johnson AD. Identification and Characterization of Wor4, a New Transcriptional Regulator of White-Opaque Switching. G3 (Bethesda). 2016. doi: 10.1534/g3.115.024885. PubMed PMID: 26772749.
111. Nie X, Liu X, Wang H, Chen J. Deletion of *EFG1* promotes *Candida albicans* opaque formation responding to pH via Rim101. Acta biochimica et biophysica Sinica. 2010;42(10):735-44. doi: 10.1093/abbs/gmq076. PubMed PMID: 20870932.

112. Srikantha T, Tsai LK, Daniels K, Soll DR. *EFG1* Null Mutants of *Candida albicans* Switch but Cannot Express the Complete Phenotype of White-Phase Budding Cells. *Journal of bacteriology*. 2000;182(6):1580-91.
113. Proft M, Struhl K. Hog1 kinase converts the Sko1-Cyc8-Tup1 repressor complex into an activator that recruits SAGA and SWI/SNF in response to osmotic stress. *Molecular Cell*. 2002;9:1307-17.
114. Schulze JM, Wang AY, Kobor MS. YEATS domain proteins: a diverse family with many links to chromatin modification and transcription. *Biochemistry and Cell Biology*. 2009;87(1):65-75. doi: 10.1139/o08-111.
115. Ene IV, Lohse MB, Vladu AV, Morschhauser J, Johnson AD, Bennett RJ. Phenotypic Profiling Reveals that *Candida albicans* Opaque Cells Represent a Metabolically Specialized Cell State Compared to Default White Cells. *MBio*. 2016;7(6). doi: 10.1128/mBio.01269-16. PubMed PMID: 27879329; PubMed Central PMCID: PMC5120136.
116. Leach MD, Farrer RA, Tan K, Miao Z, Walker LA, Cuomo CA, et al. Hsf1 and Hsp90 orchestrate temperature-dependent global transcriptional remodelling and chromatin architecture in *Candida albicans*. *Nat Commun*. 2016;7:11704. doi: 10.1038/ncomms11704. PubMed PMID: 27226156; PubMed Central PMCID: PMC4894976.
117. Ene IV, Adya AK, Wehmeier S, Brand AC, MacCallum DM, Gow NA, et al. Host carbon sources modulate cell wall architecture, drug resistance and virulence in a fungal pathogen. *Cellular microbiology*. 2012;14(9):1319-35. doi: 10.1111/j.1462-5822.2012.01813.x. PubMed PMID: 22587014; PubMed Central PMCID: PMC3465787.
118. Ene IV, Cheng SC, Netea MG, Brown AJ. Growth of *Candida albicans* Cells on the Physiologically Relevant Carbon Source Lactate Affects Their Recognition and Phagocytosis by Immune Cells. *Infect Immun*. 2013;81(1):238-48.
119. Ballou ER, Avelar GM, Childers DS, Mackie J, Bain JM, Wagener J, et al. Lactate signalling regulates fungal beta-glucan masking and immune evasion. *Nat Microbiol*. 2016;2:16238. doi: 10.1038/nmicrobiol.2016.238. PubMed PMID: 27941860.
120. Braun BR, Head WS, Wang MX, Johnson AD. Identification and Characterization of *TUP1*-Regulated Genes in *Candida albicans*. *Genetics*. 2000;156(1):31-44.
121. Elias-Villalobos A, Fernandez-Alvarez A, Ibeas JI. The general transcriptional repressor Tup1 is required for dimorphism and virulence in a fungal plant pathogen. *PLoS pathogens*. 2011;7(9):e1002235. doi: 10.1371/journal.ppat.1002235. PubMed PMID: 21909277; PubMed Central PMCID: PMC3164652.
122. Chen Y, Zhai S, Sun Y, Li M, Dong Y, Wang X, et al. MoTup1 is required for growth, conidiogenesis and pathogenicity of *Magnaporthe oryzae*. *Mol Plant Pathol*. 2015;16(8):799-810. doi: 10.1111/mp.12235. PubMed PMID: 25583028.
123. Johnk B, Bayram O, Abelman A, Heinekamp T, Mattern DJ, Brakhage AA, et al. SCF Ubiquitin Ligase F-box Protein Fbx15 Controls Nuclear Co-repressor Localization, Stress Response and Virulence of the Human Pathogen *Aspergillus fumigatus*. *PLoS pathogens*. 2016;12(9):e1005899. doi: 10.1371/journal.ppat.1005899. PubMed PMID: 27649508; PubMed Central PMCID: PMC5029927.

124. Lee H, Chang YC, Nardone G, Kwon-Chung KJ. *TUP1* disruption in *Cryptococcus neoformans* uncovers a peptide-mediated density-dependent growth phenomenon that mimics quorum sensing. *Molecular microbiology*. 2007;64(3):591-601. doi: 10.1111/j.1365-2958.2007.05666.x. PubMed PMID: 17462010.
125. Tollot M, Assmann D, Becker C, Altmüller J, Dutheil JY, Wegner CE, et al. The WOPR Protein Ros1 Is a Master Regulator of Sporogenesis and Late Effector Gene Expression in the Maize Pathogen *Ustilago maydis*. *PLoS pathogens*. 2016;12(6):e1005697. doi: 10.1371/journal.ppat.1005697. PubMed PMID: 27332891; PubMed Central PMCID: PMC4917244.
126. Homer CM, Summers DK, Goranov AI, Clarke SC, Wiesner DL, Diedrich JK, et al. Intracellular Action of a Secreted Peptide Required for Fungal Virulence. *Cell Host Microbe*. 2016;19(6):849-64. doi: 10.1016/j.chom.2016.05.001. PubMed PMID: 27212659.
127. Puig S, Ramos-Alonso L, Romero AM, Martínez-Pastor MT. The elemental role of iron in DNA synthesis and repair. *Metallomics*. 2017;9(11):1483-500. doi: 10.1039/c7mt00116a. PubMed PMID: 28879348.
128. Lan CY, Rodarte G, Murillo LA, Jones T, Davis RW, Dungan J, et al. Regulatory networks affected by iron availability in *Candida albicans*. *Mol Microbiol*. 2004;53(5):1451-69. doi: 10.1111/j.1365-2958.2004.04214.x. PubMed PMID: 15387822.
129. Singh RP, Prasad HK, Sinha I, Agarwal N, Natarajan K. Cap2-HAP complex is a critical transcriptional regulator that has dual but contrasting roles in regulation of iron homeostasis in *Candida albicans*. *J Biol Chem*. 2011;286(28):25154-70. doi: 10.1074/jbc.M111.233569. PubMed PMID: 21592964; PubMed Central PMCID: PMC3137088.
130. Baek YU, Li M, Davis DA. *Candida albicans* ferric reductases are differentially regulated in response to distinct forms of iron limitation by the Rim101 and CBF transcription factors. *Eukaryot Cell*. 2008;7(7):1168-79. doi: 10.1128/EC.00108-08. PubMed PMID: 18503007; PubMed Central PMCID: PMC2446673.
131. Pelletier B, Beaudoin J, Mukai Y, Labbe S. Fep1, an Iron Sensor Regulating Iron Transporter Gene Expression in *Schizosaccharomyces pombe*. *J Biol Chem*. 2002;277(25):22950-8.
132. Pelletier B, Beaudoin J, Philpott CC, Labbe S. Fep1 represses expression of the fission yeast *Schizosaccharomyces pombe* siderophore-iron transport system. *Nucleic Acids Research*. 2003;31(15):4332-44.
133. Pelletier B, Trott A, Morano KA, Labbe S. Functional characterization of the iron-regulatory transcription factor Fep1 from *Schizosaccharomyces pombe*. *J Biol Chem*. 2005;280(26):25146-61. doi: 10.1074/jbc.M502947200. PubMed PMID: 15866870.
134. Jung WH, Sham A, White R, Kronstad JW. Iron regulation of the major virulence factors in the AIDS-associated pathogen *Cryptococcus neoformans*. *PLoS Biol*. 2006;4(12):e410. doi: 10.1371/journal.pbio.0040410. PubMed PMID: 17121456; PubMed Central PMCID: PMC1637126.

135. Vandeputte P, Ischer F, Sanglard D, Coste AT. In vivo systematic analysis of *Candida albicans* Zn²-Cys⁶ transcription factors mutants for mice organ colonization. PLoS One. 2011;6(10):e26962. doi: 10.1371/journal.pone.0026962. PubMed PMID: 22073120; PubMed Central PMCID: PMC3205040.
136. Kumanovics A, Chen OS, Li L, Bagley D, Adkins EM, Lin H, et al. Identification of *FRA1* and *FRA2* as genes involved in regulating the yeast iron regulon in response to decreased mitochondrial iron-sulfur cluster synthesis. J Biol Chem. 2008;283(16):10276-86. doi: 10.1074/jbc.M801160200. PubMed PMID: 18281282; PubMed Central PMCID: PMC2447656.
137. Li H, Mapolelo DT, Dingra NN, Naik SG, Lees NS, Hoffman BM, et al. The yeast iron regulatory proteins Grx3/4 and Fra2 form heterodimeric complexes containing a [2Fe-2S] cluster with cysteinyl and histidyl ligation. Biochemistry. 2009;48(40):9569-81. doi: 10.1021/bi901182w. PubMed PMID: 19715344; PubMed Central PMCID: PMC2796373.
138. Kim KD, Kim HJ, Lee KC, Roe JH. Multi-domain CGFS-type glutaredoxin Grx4 regulates iron homeostasis via direct interaction with a repressor Fep1 in fission yeast. Biochem Biophys Res Commun. 2011;408(4):609-14. doi: 10.1016/j.bbrc.2011.04.069. PubMed PMID: 21531205.
139. Jacques JF, Mercier A, Brault A, Mourer T, Labbe S. Fra2 is a co-regulator of Fep1 inhibition in response to iron starvation. PLoS One. 2014;9(6):e98959. doi: 10.1371/journal.pone.0098959. PubMed PMID: 24897379; PubMed Central PMCID: PMC4045890.
140. Chaves GM, Bates S, MacCallum DM, Odds FC. *Candida albicans* *GRX2*, encoding a putative glutaredoxin, is required for virulence in a murine model. Genet Mol Res. 2007;6(4):1051-63.
141. Zhang D, Dong Y, Yu Q, Kai Z, Zheng M, Jia C, et al. Function of glutaredoxin 3 (Grx3) in oxidative stress response caused by iron homeostasis disorder in *Candida albicans*. Future Microbiol. 2017;12(15):1397-412.
142. Enjalbert B, MacCallum DM, Odds FC, Brown AJ. Niche-specific activation of the oxidative stress response by the pathogenic fungus *Candida albicans*. Infect Immun. 2007;75(5):2143-51. doi: 10.1128/IAI.01680-06. PubMed PMID: 17339352; PubMed Central PMCID: PMC1865731.
143. Dlouhy AC, Beaudoin J, Labbe S, Outten CE. *Schizosaccharomyces pombe* Grx4 regulates the transcriptional repressor Php4 via [2Fe-2S] cluster binding. Metallomics. 2017;9(8):1096-105. doi: 10.1039/c7mt00144d. PubMed PMID: 28725905; PubMed Central PMCID: PMC5595146.
144. Spiller MP, Ang SK, Ceh-Pavia E, Fisher K, Wang Q, Rigby SE, et al. Identification and characterization of mitochondrial Mia40 as an iron-sulfur protein. Biochem J. 2013;455(1):27-35.
145. Flint DH, Emptage MH, Guest JR. Fumarase a from *Escherichia coli*: purification and characterization as an iron-sulfur cluster containing enzyme. Biochemistry. 1992;31(42):10331-7.
146. Lill R, Hoffmann B, Molik S, Pierik AJ, Rietzschel N, Stehling O, et al. The role of mitochondria in cellular iron-sulfur protein biogenesis and iron metabolism. Biochim

- Biophys Acta. 2012;1823(9):1491-508. doi: 10.1016/j.bbamcr.2012.05.009. PubMed PMID: 22609301.
147. Feliciano PR, Drennan CL, Nonato MC. Crystal structure of an Fe-S cluster-containing fumarate hydratase enzyme from *Leishmania major* reveals a unique protein fold. Proc Natl Acad Sci U S A. 2016;113(35):9804-9. doi: 10.1073/pnas.1605031113. PubMed PMID: 27528683; PubMed Central PMCID: PMC5024648.
148. Amillet J-M, Buisson N, Labbe-Bois R. Positive and negative elements involved in the differential regulation by heme and oxygen of the HEM13 gene (coproporphyrinogen oxidase) in *Saccharomyces cerevisiae*. Curr Genet. 1995;28(6):503-11.
149. Frey AG, Palenchar DJ, Wildemann JD, Philpott CC. A Glutaredoxin-BolA Complex Serves as an Iron-Sulfur Cluster Chaperone for the Cytosolic Cluster Assembly Machinery. J Biol Chem. 2016;291(43):22344-56. doi: 10.1074/jbc.M116.744946. PubMed PMID: 27519415; PubMed Central PMCID: PMC5077177.
150. Thomas PD, Campbell MJ, Kejariwal A, Mi H, Karlak B, Daverman R, et al. PANTHER: A Library of Protein Families and Subfamilies Indexed by Function. Genome Res. 2003;13(9):2129-41. doi: 10.1101/gr.772403. PubMed Central PMCID: PMC403709
151. Thomas PD, Kejariwal A, Guo N, Mi H, Campbell MJ, Muruganujan A, et al. Applications for protein sequence-function evolution data: mRNA/protein expression analysis and coding SNP scoring tools. Nucleic Acids Research. 2006;34(Web Server):W645-W50. doi: 10.1093/nar/gkl229.
152. Fuss JO, Tsai CL, Ishida JP, Tainer JA. Emerging critical roles of Fe-S clusters in DNA replication and repair. Biochim Biophys Acta. 2015;1853(6):1253-71. doi: 10.1016/j.bbamcr.2015.01.018. PubMed PMID: 25655665; PubMed Central PMCID: PMC4576882.
153. Beinert H. Iron-sulfur proteins: ancient structures, still full of surprises. J Biol Inorg Chem. 2000;5(1):2-15.
154. Andreini C, Rosato A, Banci L. The Relationship between Environmental Dioxygen and Iron-Sulfur Proteins Explored at the Genome Level. PLoS One. 2017;12(1):e0171279. doi: 10.1371/journal.pone.0171279. PubMed PMID: 28135316; PubMed Central PMCID: PMC5279795.
155. Paul VD, Lill R. Biogenesis of cytosolic and nuclear iron-sulfur proteins and their role in genome stability. Biochim Biophys Acta. 2015;1853(6):1528-39. doi: 10.1016/j.bbamcr.2014.12.018. PubMed PMID: 25583461.
156. Gari K, Ortiz AML, Borel V, Flynn H, Skehel JM, Boulton SJ. MMS19 Links Cytoplasmic Iron-Sulfur Cluster Assembly to DNA Metabolism. Science. 2012;337(6091):243-5.
157. Stehling O, Vashisht AA, Mascarenhas J, Jonsson ZO, Sharma T, Netz DJA, et al. MMS19 Assembles Iron-Sulfur Proteins Required for DNA Metabolism and Genomic Integrity. Science. 2012;337(6091):195-9.
158. Veatch JR, McMurray MA, Nelson ZW, Gottschling DE. Mitochondrial dysfunction leads to nuclear genome instability via an iron-sulfur cluster defect. Cell.

- 2009;137(7):1247-58. doi: 10.1016/j.cell.2009.04.014. PubMed PMID: 19563757; PubMed Central PMCID: PMCPMC2759275.
159. Pijuan J, Maria C, Herrero E, Belli G. Impaired mitochondrial Fe-S cluster biogenesis activates the DNA damage response through different signaling mediators. *J Cell Sci.* 2015;128(24):4653-65. doi: 10.1242/jcs.178046. PubMed PMID: 26567217.
160. Brault A, Mourer T, Labbe S. Molecular basis of the regulation of iron homeostasis in fission and filamentous yeasts. *IUBMB Life.* 2015;67(11):801-15. doi: 10.1002/iub.1441. PubMed PMID: 26472434.
161. Xu N, Cheng X, Yu Q, Qian K, Ding X, Liu R, et al. Aft2, a novel transcription regulator, is required for iron metabolism, oxidative stress, surface adhesion and hyphal development in *Candida albicans*. *PLoS One.* 2013;8(4):e62367. doi: 10.1371/journal.pone.0062367. PubMed PMID: 23626810; PubMed Central PMCID: PMCPMC3633901.
162. Prakash S, Prakash L. Nucleotide excision repair in yeast. *Mutat Res.* 2000;451(1-2):13-24.
163. Dib L, Hayek P, Sadek H, Beyrouthy B, Khalaf RA. The *Candida albicans* Ddr48 protein is essential for filamentation, stress response, and confers partial antifungal drug resistance. *Med Sci Monit.* 2008;14(6):113-21.
164. Cleary IA, MacGregor NB, Saville SP, Thomas DP. Investigating the function of Ddr48p in *Candida albicans*. *Eukaryot Cell.* 2012;11(6):718-24. doi: 10.1128/EC.00107-12. PubMed PMID: 22523369; PubMed Central PMCID: PMCPMC3370460.
165. Sanvisens N, Bano MC, Huang M, Puig S. Regulation of ribonucleotide reductase in response to iron deficiency. *Mol Cell.* 2011;44(5):759-69. doi: 10.1016/j.molcel.2011.09.021. PubMed PMID: 22152479; PubMed Central PMCID: PMCPMC3240860.
166. Knight SAB, Lesuisse E, Stearman R, Klausner RD, Dancis A. Reductive iron uptake by *Candida albicans*: role of copper, iron and the *TUP1* regulator. *Microbiology.* 2002;148(1):29-40.
167. Tanner FWJ, Vojnovich C, Van Lanen JM. Riboflavin production by *Candida* species. *Science.* 1945;101(2616):180-1.
168. Crossley RA, Gaskin DJ, Holmes K, Mulholland F, Wells JM, Kelly DJ, et al. Riboflavin biosynthesis is associated with assimilatory ferric reduction and iron acquisition by *Campylobacter jejuni*. *Appl Environ Microbiol.* 2007;73(24):7819-25. doi: 10.1128/AEM.01919-07. PubMed PMID: 17965203; PubMed Central PMCID: PMCPMC2168145.
169. Dmytruk KV, Voronovsky AY, Sibirny AA. Insertion mutagenesis of the yeast *Candida famata* (*Debaryomyces hansenii*) by random integration of linear DNA fragments. *Curr Genet.* 2006;50(3):183-91. doi: 10.1007/s00294-006-0083-0. PubMed PMID: 16770625.
170. Rodríguez-Celma J, Schmidt W. Reduction-based iron uptake revisited. *Plant Signaling & Behavior.* 2014;8(11):e26116. doi: 10.4161/psb.26116.
171. Kispal G, Sipos K, Lange H, Fekete Z, Bedekovics T, Janáky T, et al. Biogenesis of cytosolic ribosomes requires the essential iron-sulphur protein Rli1p and mitochondria. *EMBO J.* 2005;24:589-98. doi: 10.1038/.

172. Yarunin A, Panse VG, Petfalski E, Dez C, Tollervey D, Hurt EC. Functional link between ribosome formation and biogenesis of iron–sulfur proteins. *EMBO J*. 2005;24:580-8. doi: 10.1038/.
173. Khoshnevis S, Gross T, Rotte C, Baierlein C, Ficner R, Krebber H. The iron-sulphur protein RNase L inhibitor functions in translation termination. *EMBO Rep*. 2010;11(3):214-9. doi: 10.1038/embor.2009.272. PubMed PMID: 20062004; PubMed Central PMCID: PMC2838684.
174. Inigo S, Durand AN, Ritter A, Le Gall S, Termathe M, Klassen R, et al. Glutaredoxin GRXS17 Associates with the Cytosolic Iron-Sulfur Cluster Assembly Pathway. *Plant Physiol*. 2016;172(2):858-73. doi: 10.1104/pp.16.00261. PubMed PMID: 27503603; PubMed Central PMCID: PMC4707072.
175. Han GW, Yang XL, McMullan D, Chong YE, Krishna SS, Rife CL, et al. Structure of a tryptophanyl-tRNA synthetase containing an iron-sulfur cluster. *Acta Crystallogr Sect F Struct Biol Cryst Commun*. 2010;66(Pt 10):1326-34. doi: 10.1107/S1744309110037619. PubMed PMID: 20944229; PubMed Central PMCID: PMC2954223.
176. Page WJ, Mehrotra M, Vande Woestyne M, Tindale AE, Kujat Choy SL, Macyk AS, et al. Iron-regulated phenylalanyl-tRNA synthetase activity in *Azotobacter vinelandi*. *FEMS Microbiol Lett*. 2003;218(1):15-21.