Analysis of the roles of a monothiol glutaredoxin and glutathione synthetase in the virulence of the AIDS-associated pathogen Cryptococcus neoformans

by

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Abstract

_Cryptococcus neoformans_ is a fungal pathogen and the causative agent of meningoencephalitis in immunocompromised and AIDS patients. Iron acquisition and the maintenance of iron homeostasis in _C. neoformans_ are important aspects of virulence. Here, the monothiol glutaredoxin Grx4 was identified as a binding partner of Cir1, the master regulator of iron-responsive genes and virulence factor expression in _C. neoformans_. We confirmed that Grx4 binds Cir1 in vitro and in a yeast two-hybrid assay. RNA-Seq performed on the _grx4_ and _cir1_ mutants, and the WT strain, under low or high iron conditions identified genes involved in iron metabolism and expanded the concept that Grx4 plays a central role in iron homeostasis. A _grx4_ mutant, with deletion of the glutaredoxin domain, displayed iron-related phenotypes similar to those of a _cir1_ mutant, including elevated activity for cell surface reductases, sensitivity to high iron levels and increased susceptibility to phleomycin. Importantly it was shown that a _grx4_ mutant was avirulent in a mouse model of infection. We also observed that after 48 hour of starvation _grx4_ mutant had a growth defect in an iron-supplemented media. Interestingly, the growth defect of the _grx4_ mutant was rescued by exogenous GSH. We therefore further investigated the role of GSH in _C. neoformans_. Deletion of the _GSH2_ gene encoding the second enzyme in GSH biosynthesis resulted in a significant decrease in intracellular GSH levels in _gsh2_ mutants compared to the WT. This also resulted in loss of elaboration of major virulence factors including production of capsule and melanin, growth at host body temperature, and enhanced susceptibility to antifungal drugs. In conclusion GSH is required for expression of major virulence factors and defense against iron starvation-induced stress. GSH also influenced cell wall integrity. These results indicate that GSH
plays a major role in the virulence of *C. neoformans*, and suggest a crosstalk between Grx4 and GSH as components of iron homeostasis and virulence expression. Overall, our findings provide further understanding of the regulation of virulence in *C. neoformans* and suggest novel drug targets for anticryptococcal therapy.
Preface

Relative contributions of all collaborators

The experimental design of the research outlined in this thesis was achieved through collaboration between Rodgoun Attarian and Dr. James W. Kronstad. R. Attarian conducted the experiments, and the people who have contributed to this work are identified below and acknowledged throughout the thesis.

The work presented in Chapter 2 is in preparation for publication as a manuscript entitled “Monothiol Glutaredoxin-4 is required for iron homeostasis and virulence in the AIDS-associated fungal pathogen Cryptococcus neoformans”. The details of contributions to this work are as follows: Dr. Horacio Bach contributed to this study by identifying Grx4 by mass-spectrometry. Dr. Yossef Av-Gay provided access to ALPHAScreen technology. Dr. Patricia Missall and Dr. Jennifer Lodge provided the grx4 mutant. Dr. Melissa Caza performed the virulence assays and determined the fungal loads in mice tissues. Dr. Daniel Croll performed gene ontology enrichment analyses on RNA-seq data. Dr. Guanggan Hu generated the CIR1- pDEST™32 and GRX4-pDEST™22 strains in E.coli for Y2H analysis, and the constructs for deletion of the entire GRX4 locus in the diploid strains. Dr. Alex Idnurm provided the diploid strain AI187. Dr. Alina Chan helped with spore isolation and Dr. Nigel O’Neil assisted in interpretation of data from sporulation progeny of grx4 mutant in the diploid background. A Work Learn student, Celine Chan, performed PCR on the generated progeny.

The work presented in Chapter 3 is in preparation for publication in a manuscript entitled “Glutathione synthetase GSH2 is indispensable for growth under low iron condition and virulence in Cryptococcus neoformans”. Dr. James W. Kronstad suggested
the idea to characterize the role of GSH synthesis. The work in Chapter 3 was performed by R. Attarian, and Dr. Guanggan Hu generated constructs for deletion of GSH2.

Publications arising from graduate work


- **Attarian R**, Hu G, Kronstad J. *(In preparation).* Glutathione synthetase Gsh2 is indispensible for growth under low iron condition and virulence in *Cryptococcus neoformans.*

University of British Columbia Ethics Board approval

The protocol for the virulence assays was approved by the University of British Columbia’s Committee on Animal Care (protocol A13-0093).
Table of Contents

Abstract ......................................................................................................................................................... ii
Preface ................................................................................................................................................................ iv
Table of Contents ............................................................................................................................................... vi
List of Tables .................................................................................................................................................... xiv
List of Figures .................................................................................................................................................. xvi
List of Abbreviations ....................................................................................................................................... xix
Acknowledgements ........................................................................................................................................ xxv
Dedication ......................................................................................................................................................... xxvi

Chapter 1: Introduction ..................................................................................................................................... 1

1.1 Cryptococcus neoformans .......................................................................................................................... 1
  1.1.1 Cryptococcal disease .............................................................................................................................. 1
  1.1.2 Cryptococcal meningoencephalitis ...................................................................................................... 2
  1.1.3 The burden of cryptococcal infection and HIV .................................................................................. 2
  1.1.4 Treatment ............................................................................................................................................. 3

1.2 Virulence features of C. neoformans .......................................................................................................... 3
  1.2.1 Growth at 37°C ..................................................................................................................................... 3
  1.2.2 Polysaccharide capsule ......................................................................................................................... 4
  1.2.3 Melanin ................................................................................................................................................. 6
  1.2.4 Other virulence traits .......................................................................................................................... 7
    1.2.4.1 Urease ............................................................................................................................................. 7
    1.2.4.2 Phospholipase ............................................................................................................................ 8
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.2.4.3</td>
<td>Oxidative defenses</td>
<td>8</td>
</tr>
<tr>
<td>1.2.4.4</td>
<td>Secreted vesicles</td>
<td>9</td>
</tr>
<tr>
<td>1.2.4.5</td>
<td>Nutrient acquisition</td>
<td>10</td>
</tr>
<tr>
<td>1.2.4.5.1</td>
<td>Iron</td>
<td>11</td>
</tr>
<tr>
<td>1.3</td>
<td>Iron homeostasis in eukaryotic models: <em>S. cerevisiae</em> and <em>S. pombe</em></td>
<td>13</td>
</tr>
<tr>
<td>1.3.1</td>
<td>Iron homeostasis in <em>S. cerevisiae</em></td>
<td>13</td>
</tr>
<tr>
<td>1.3.2</td>
<td>Iron homeostasis in <em>S. pombe</em></td>
<td>15</td>
</tr>
<tr>
<td>1.4</td>
<td>Monothiol glutaredoxins</td>
<td>16</td>
</tr>
<tr>
<td>1.4.1</td>
<td>Roles of monothiol Grxs</td>
<td>18</td>
</tr>
<tr>
<td>1.4.1.1</td>
<td>Role of monothiol Grx3/4 in regulation of Aft1 in yeast</td>
<td>18</td>
</tr>
<tr>
<td>1.4.1.2</td>
<td>Role of monothiol Grx4 in the regulation of Fep1 in fission yeast</td>
<td>19</td>
</tr>
<tr>
<td>1.4.1.3</td>
<td>Role of monothiol Grx4 in regulation of Php4 in fission yeast</td>
<td>20</td>
</tr>
<tr>
<td>1.4.1.4</td>
<td>Monothiol CGFS Grxs contain a labile Fe–S cluster</td>
<td>20</td>
</tr>
<tr>
<td>1.5</td>
<td>Glutathione</td>
<td>21</td>
</tr>
<tr>
<td>1.5.1</td>
<td>Roles of glutathione</td>
<td>22</td>
</tr>
<tr>
<td>1.5.1.1</td>
<td>A vital function of glutathione in iron homeostasis</td>
<td>23</td>
</tr>
<tr>
<td>1.5.1.1.1</td>
<td>Role of glutathione in iron homeostasis in fungi</td>
<td>24</td>
</tr>
<tr>
<td>1.6</td>
<td>Iron uptake in <em>C. neoformans</em></td>
<td>25</td>
</tr>
<tr>
<td>1.6.1</td>
<td>The high-affinity iron uptake pathway</td>
<td>26</td>
</tr>
<tr>
<td>1.6.2</td>
<td>Low-affinity iron uptake pathway</td>
<td>26</td>
</tr>
<tr>
<td>1.6.3</td>
<td>Siderophore-bound iron transport</td>
<td>27</td>
</tr>
<tr>
<td>1.6.4</td>
<td>Heme uptake</td>
<td>28</td>
</tr>
<tr>
<td>1.6.5</td>
<td>Cir1: Master regulator of iron</td>
<td>28</td>
</tr>
</tbody>
</table>
Chapter 2: Monothiol glutaredoxin-4 is required for iron homeostasis and virulence in the AIDS-associated pathogen Cryptococcus neoformans.................................34

2.1 Introduction ........................................................................................................34

2.2 Materials and methods .........................................................................................37

2.2.1 Yeast strains, plasmids and growth media.........................................................37

2.2.2 Construction of strains ......................................................................................39

2.2.2.1 Deletion of the conserved Grx domain to create a grx4 mutant and generation of grx4Δ::GRX4 complemented strain .........................................................41

2.2.2.2 Genetic analysis of GRX4 in a haploid and diploid C. neoformans background ..................................................................................................................42

2.2.3 In silico protein analysis ......................................................................................44

2.2.4 Gene cloning, protein expression and purification..............................................44

2.2.5 Protein–protein interaction ...............................................................................45

2.2.5.1 AlphaScreen™ binding assays .......................................................................45

2.2.5.2 Yeast two-hybrid assay ..................................................................................46

2.2.6 Iron-related phenotypic analysis .......................................................................47

2.2.7 Virulence factor assays .....................................................................................47

2.2.8 Analysis of growth in liquid media ...................................................................48

2.2.9 Virulence assay ..................................................................................................49

2.2.10 Ethics statement ................................................................................................50
2.2.11 Statistical analysis ........................................................................................................50
2.2.12 RNA-Seq library preparation and bioinformatics ......................................................50
2.2.13 Quantitative Real-Time PCR .....................................................................................52
2.3 Results ..................................................................................................................................53
2.3.1 The monothiol glutaredoxin Grx4 binds Cir1.................................................................53
   2.3.1.1 Grx4 was identified as a potential Cir1-associated protein.................................53
   2.3.1.2 Production of recombinant Grx4 and Cir1 in E. coli for interaction studies...........54
   2.3.1.3 Grx4 binds Cir1 in an ALPHAScreen® protein-protein interaction assay..............57
   2.3.1.4 Grx4 binds Cir1 in a yeast two-hybrid assay..........................................................61
2.3.2 A defect in Grx4 impacts iron homeostasis and virulence............................................63
   2.3.2.1 Identification of a conserved C-terminal glutaredoxin domain in Grx4 in C. neoformans ........................................................................................................................................63
   2.3.2.2 A grx4 deletion mutant has iron-related phenotypes.............................................65
   2.3.2.3 Grx4 is required for inhibition of iron uptake in the presence of iron......71
   2.3.2.4 Grx4 influences the virulence of C. neoformans......................................................74
      2.3.2.4.1 The grx4 mutant displays virulence-related phenotypes..............................74
      2.3.2.4.2 Deletion of grx4 abrogates virulence in a mouse model of cryptococcosis ......76
2.3.3 A pilot transcriptomic analysis using RNA-seq provided further insight into role of Grx4 and also revealed complexities in the transcript structure of GRX4 .................................................................79
2.3.3.1 Grx4 is required for broad transcriptional remodeling in response to iron in *C. neoformans*.

2.3.3.1.1 Categories of differentially expressed genes based on GO terms associated with molecular function in the *grx4* mutant compared to WT cells grown under the iron-deplete condition.

2.3.3.1.2 Categories of differentially expressed genes based on GO terms associated with molecular function in the *grx4* mutant compared to WT cells grown under the iron-replete condition.

2.3.3.2 Analysis of the data from the pilot RNA-seq analysis identified several putative transcripts associated with the *GRX4* locus.

2.3.3.3 Analysis of *GRX4* essentiality.

2.3.3.3.1 Deletion of the entire *GRX4* including the N-terminal region in a stable diploid (strain AI187) background shows that the N-terminal region of *GRX4* is not essential.

2.3.3.3.2 Genetic segregation analysis of heterozygous auxotrophic markers in progeny isolated from a stable diploid with a confirmed deletion of *GRX4*.

2.4 Discussion.

2.4.1 The conserved C-terminal monothiol Grx domain of Grx4 physically binds to the N-terminal region of Cir1.

2.4.2 The *grx4* mutant displays iron-related and virulence-related phenotypes.
2.4.3 Grx4 has a central role in maintenance of iron homeostasis in *C. neoformans*  ........................................................................................................98

2.4.4 Grx4 is required for virulence in *C. neoformans*.........................................................................................100

2.4.5 The N-terminal of Grx4 is not essential and exists in some but not all transcript isoforms from the *GRX4* locus.........................................................................................................................101

Chapter 3: Analysis of the roles of glutathione synthetase in *Cryptococcus neoformans* ........................................................................................................................................................................104

3.1 Introduction ..........................................................................................................................................................104

3.2 Materials and methods .......................................................................................................................................110

3.2.1 *In silico* protein analysis ...............................................................................................................................110

3.2.2 Yeast strains, plasmids and growth media .................................................................................................110

3.2.3 Generation of *gsh2* mutants and a *gsh2Δ::GSH2* complemented strain ..........111

3.2.4 Phenotypic and biochemical analyses ..................................................................................................113

3.2.4.1 Growth Assay ...............................................................................................................................................113

3.2.4.2 Quantification of the intracellular content of total (reduced and oxidized or GSHt) glutathione.................................................................................................................................113

3.2.4.3 Capsule formation, melanin production, and growth at 37°C ...............115

3.2.4.4 Stress and drug response assays .................................................................................................................116

3.2.4.5 Sensitivity to inhibitors of the mitochondria electron transport chain.116

3.3 Results .............................................................................................................................................................117

3.3.1 GSH synthetase encoded by *GSH2* in *C. neoformans* belongs to the eukaryotic glutathione synthetase (eu-GS) family in the ATP-grasp superfamily.................117
3.3.2 GSH synthetase (Gsh2) is dispensable for growth under normal conditions in C. neoformans

3.3.3 gsh2 mutants are sensitive to iron starvation and require exogenous GSH for growth on minimal or iron-replete media after iron starvation

3.3.4 The gsh2 mutants have reduced intracellular total glutathione (GSHt) compared to the WT strain

3.3.5 gsh2 is involved in multi-stress resistance

3.3.6 Lack of Gsh2 affects virulence factors in C. neoformans

3.3.7 gsh2 mutants display increased susceptibility to antifungal drugs

3.3.8 gsh2 mutants display subtle susceptibility to mitochondria inhibitors

3.4 Discussion

3.4.1 GSH synthetase is dispensable for growth under normal growth conditions

3.4.2 Phenotypic analysis of gsh2 mutants suggest an interplay between GSH homeostasis and iron homeostasis in C. neoformans

3.4.3 Phenotypic characterization of gsh2 mutants revealed that Gsh2 is required for a plethora of functions in C. neoformans

Chapter 4: Discussion

4.1 Iron Homeostasis in C. neoformans

4.2 Monothiol Grx4 is required for iron homeostasis and virulence in C. neoformans

4.3 GSH is involved in stress response and virulence in C. neoformans

4.4 Conclusion

4.5 Key areas for future directions
4.5.1 A Proposed model of Grx4-Cir1 interaction ..................................................154
4.5.2 A proposed crosstalk model for GSH functions .............................................156
4.6 Future experiments .............................................................................................161
4.6.1 Studying the interaction of Grx4 with Cir1 in *C. neoformans* .......................161
4.6.2 Investigating Grx4 capacity to assemble Fe-S clusters ................................161
4.6.3 Further understanding of the transcript structure of the *GRX4* locus ........162
4.6.4 Investigation of reversible S-glutathionylation of Cir1 .................................163
4.6.5 Construction and phenotypic characterization of GSH transporter deletion mutants ..................................................................................................................................................164
4.6.6 Investigation of Grx4 and GSH crosstalk in *C. neoformans* .......................164

References ..............................................................................................................166

Appendices .............................................................................................................203

Appendix A .............................................................................................................203

Appendix B .............................................................................................................217
List of Tables

Table 2.1: Strains used in Chapter 2 ........................................................................................................38
Table 2.2: Primers sequences for strain construction ...............................................................................39
Table 2.3: Primer sequences for quantitative qRT-PCR .......................................................................53
Table 3.1: Strains used in Chapter 3 .......................................................................................................111
Table 3.2: Primer sequences for strain construction ...............................................................................111
Table A1: List of proteins identified in Cir1 binding assay identified by Mass-Spectrometry in an experiment performed by Dr. Horacio Bach ................................................................................203
Table A2: Categories of differentially expressed genes based on GO terms associated with molecular function in the grx4 mutant compared to WT cells grown under the iron-deplete condition ................................................................................................................................................206
Table A3: Categories of differentially expressed genes based on GO terms associated with molecular function in the grx4 mutant compared to WT cells grown under iron-replete condition ................................................................................................................................................208
Table A4: Categories of differentially expressed genes based on GO terms associated with molecular function with similar patterns of expression in the grx4 and cir1 mutants compared to WT cells grown under iron-deplete condition ................................................................................................................................................210
Table A5: Categories of differentially expressed genes based on GO terms associated with molecular function with similar patterns of expression in the grx4 and cir1 mutants compared to WT cells grown under iron-replete condition ................................................................................................................................................213
Table A6: Categories of differentially expressed genes based on GO terms associated with molecular function with distinct patterns of expression in the grx4 or cir1 mutants compared to WT cells grown under iron-deplete condition ................................................................................................................................................215
Table A7: Categories of differentially expressed genes based on GO terms associated with molecular function with distinct patterns of expression in the *grx4* or *cir1* mutants compared to WT cells grown under iron-replete condition

---

216
List of Figures

Figure 1.1: Microscopy image of India ink staining showing the characteristic halo caused by the presence of the polysaccharide capsule of *C. neoformans* ..................................................5

Figure 1.2: The dark pigment melanin in the cell wall in presence of laccase substrates such as L-3,4-dihydroxyphenylalanine (L-DOPA)........................................................................................................7

Figure 1.3: Reactive oxygen species (ROS) are generated in the Fenton reaction in the presence of iron and oxygen..................................................................................................................11

Figure 1.4: Comparison of domain structures across species for monothiol Grxs........17

Figure 1.5: Components of the iron network in *C. neoformans*.................................30

Figure 2.1: Construction of grx4 mutant and complemented *grx4Δ::GRX4* strain, and confirmation of *grx4* mutant by Southern Hybridization ..........................................................43

Figure 2.2: Identified peptides obtained from MS analysis............................................54

Figure 2.3: Purification of the His6-tagged Grx4 polypeptide.......................................55

Figure 2.4: Purification of Cir1 tagged with the maltose binding protein (MBP).........57

Figure 2.5: Overview of the Grx4-Cir1 Alphascreen assay..........................................58

Figure 2.6: Binding between Cir1 and Grx4 detected by ALPHAScreen analysis..........60

Figure 2.7: Grx4 physically interacts with Cir1 in a yeast two-hybrid assay..............62

Figure 2.8: Domain structure of *C. neoformans* Grx4........................................64

Figure 2.9: A grx4 mutant displays iron-related phenotypes similar to the cir1 mutant..67

Figure 2.10: Grx4 is required for growth in iron-depleted media supplemented with FeCl3........................................................................................................................................70

Figure 2.11: Addition of GSH restored growth of grx4 mutant under iron-replete conditions ........................................................................................................................................71
Figure 2.12: Grx4 has a negative regulatory influence on the transcript levels for iron acquisition functions.................................................................73

Figure 2.13: A grx4 mutant does not express virulence-related traits............................75

Figure 2.14: Loss of GRX4 abolishes virulence.............................................................76

Figure 2.15: Determination of fungal loads in mice tissue............................................78

Figure 2.16: Enrichment of genes differentially expressed in RNA-Seq analysis of the grx4 mutant and the WT strain under the iron-deplete condition...............................82

Figure 2.17: Enrichment of genes differentially expressed in the RNA-Seq analysis of the grx4 mutant and the WT strain in the iron-replete condition..................................84

Figure 2.18: Exon–intron organisation of C. neoformans GRX4 locus............................97

Figure 2.19: Full deletion of GRX4 in a stable H99 diploid background.........................90

Figure 2.20: Analysis of progeny..................................................................................92

Figure 3.1: Structure of GSH....................................................................................105

Figure 3.2: Biological functions associated with GSH................................................107

Figure 3.3: Enzymatic pathway for GSH synthesis in eukaryotes...............................109

Figure 3.4: Domain structure and blastp analysis of Gsh2 in C. neoformans .............118

Figure 3.5: Gsh2 is dispensable for growth under normal conditions in C. neoformans..119

Figure 3.6: Growth phenotypes of gsh2 mutants in YNB and iron-replete media after iron starvation....................................................................................122

Figure 3.7: Total glutathione (GSHt) levels in the gsh2 mutants.................................125

Figure 3.8: The gsh2 mutants have increased susceptibility to salt stress and thiol oxidizing agent diamide.................................................................127
Figure 3.9: The gsh2 mutants are impaired in virulence-related traits ..........................129

Figure 3.10: Deletion of GSH2 Increases susceptibility to antifungal agents..................130

Figure 3.11: gsh2 mutants display a subtle susceptibility to ETC inhibitors......................131

Figure 4.1: A schematic model of Grx4-Cir1 interaction ................................................156

Figure 4.2: Schematics of the proposed functional crosstalk among GSH, iron homeostasis
and cellular functions in C. neoformans........................................................................160

Figure A.1: Functional categorization of Cir1 potential binding partners......................205

Figure B.1: Deletion of GSH2 in gDNA extracted from 3 individual gsh2Δ mutants and re-
integration of GSH2 in locus in gDNA extracted from gsh2Δ::GSH2 in C. neoformans H99
background was confirmed by PCR.............................................................................217
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>3HAA</td>
<td>3-hydroxyanthranilic acid</td>
</tr>
<tr>
<td>AAs</td>
<td>amino acids</td>
</tr>
<tr>
<td>AFT1</td>
<td>iron-regulated activator of transcription</td>
</tr>
<tr>
<td>AIDS</td>
<td>acquired immune deficiency syndrome</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>BBB</td>
<td>blood brain barrier</td>
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<tr>
<td>BLAST</td>
<td>basic local alignment search tool</td>
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<td>BPS</td>
<td>bathophenanthroline disulfonate</td>
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<td>BSA</td>
<td>bovine serum albumin</td>
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<td>cAMP</td>
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<tr>
<td>Cir1</td>
<td><em>Cryptococcus</em> iron regulator 1</td>
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<td>CNS</td>
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<td>HIS</td>
<td>histidine</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HYG</td>
<td>hygromycin</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>ISC</td>
<td>Fe-S cluster assembly machinery</td>
</tr>
<tr>
<td>K_d</td>
<td>dissociation constant</td>
</tr>
<tr>
<td>L-DOPA</td>
<td>L-3,4-dihydrophenylalanine</td>
</tr>
<tr>
<td>LIM</td>
<td>low iron medium</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>MBP</td>
<td>maltose binding protein</td>
</tr>
<tr>
<td>MIT</td>
<td>Massachusetts Institute of Technology</td>
</tr>
<tr>
<td>MP</td>
<td>mannoprotein</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NAT</td>
<td>nourseothricin</td>
</tr>
<tr>
<td>NEO</td>
<td>neomycin</td>
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<td>NTA</td>
<td>nitrilotriacetic acid</td>
</tr>
<tr>
<td>N-terminal</td>
<td>amino-terminal</td>
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<tr>
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<td>phosphate-buffered saline</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
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<tr>
<td>PLB</td>
<td>phospholipase B</td>
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<tr>
<td>qRT-PCR</td>
<td>quantitative real time polymerase chain reaction</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
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</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>rRNA</td>
<td>ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>RNA-seq</td>
<td>ribonucleic acid sequencing</td>
</tr>
<tr>
<td>RNI</td>
<td>Reactive nitrogen intermediates</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>SAGE</td>
<td>serial analysis of gene expression</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
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<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<td>salicylhydroxamic acid</td>
</tr>
<tr>
<td>Sit1</td>
<td>siderophore iron transporter</td>
</tr>
<tr>
<td>Sod1</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>sp.</td>
<td>species</td>
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<tr>
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<td>trichloroacetic acid</td>
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<tr>
<td>TRX</td>
<td>thioredoxin</td>
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<tr>
<td>TTC</td>
<td>2,3,5-Triphenyl tetrazolium chloride</td>
</tr>
<tr>
<td>URA</td>
<td>uracil</td>
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<tr>
<td>UV</td>
<td>Ultra Violet</td>
</tr>
<tr>
<td>var.</td>
<td>variety</td>
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<tr>
<td>WT</td>
<td>wild-type</td>
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<tr>
<td>YNB</td>
<td>yeast nitrogen base</td>
</tr>
<tr>
<td>Y2H</td>
<td>yeast-two-hybrid</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<td>---------</td>
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<tr>
<td>YPD</td>
<td>yeast extract peptone dextrose</td>
</tr>
<tr>
<td>ZF</td>
<td>zinc finger</td>
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</tbody>
</table>
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To Dania, Houshang,

& 20 years of Babken Avak Babayan's solitude
Chapter 1: Introduction

1.1 *Cryptococcus neoformans*

*Cryptococcus neoformans* is an opportunistic fungal pathogen that has been isolated from diverse environmental niches including several different tree species (e.g., eucalyptus trees, Douglas fir) and soil that is contaminated with the excreta of birds, particularly pigeons (J. A. Barnett, 2010, A. Botes et al., 2009, K. L. Buchanan and J. W. Murphy, 1998, N. P. Govender et al., 2011, H. C. Gugnani et al., 2005, S. E. Kidd et al., 2003, X. Lin et al., 2006, A. Medeiros Ribeiro et al., 2006, K. Nielsen et al., 2007, N. Refojo et al., 2009). Importantly, *C. neoformans*, along with a related species *C. gattii*, is also the causative agent of cryptococcosis, an often-fatal fungal disease of humans and other animals (K. H. Bartlett et al., 2008, L. M. Hoang et al., 2004, N. Saul et al., 2008).

1.1.1 Cryptococcal disease

Infection with *C. neoformans* is one of the leading causes of morbidity and mortality among patients afflicted with HIV/AIDS. Specifically, it has been estimated that cryptococcosis is responsible for an estimated 1 million cases of meningoencephalitis and over 600,000 deaths annually, mostly among HIV-infected patients in sub-Saharan Africa (B. J. Park et al., 2009). Patients undergoing immunosuppressive therapy, such as transplant or cancer patients are also at risk for cryptococcal disease (P. G. Pappas, 2013, M. Revest et al., 2006). Individuals are usually colonized with *C. neoformans* through inhalation of either spores or desiccated yeast cells from the environment, although spores have yet to be observed outside the laboratory setting (R. Velagapudi et al., 2009). Infection with *C. neoformans* is most commonly initiated when fungal cells are inhaled and deposited into the alveoli (B. C. Fries et al., 2001). In immunocompetent individuals, the
infection is often cleared by the host’s immune system or it can become latent upon containment by macrophages. In immunocompromised patients, however, the pathogen can proliferate, cross the blood-brain barrier and progress to the central nervous system to cause meningoencephalitis (K. Vu et al., 2014).

1.1.2 Cryptococcal meningoencephalitis

An important aspect of cryptococcal disease is the ability of this fungus to proliferate in the brain and cerebrospinal fluid leading to cryptococcal meningoencephalitis; this is the most frequent and fatal manifestation of cryptococcosis in HIV-infected individuals (B. C. Fries et al., 2005, K. J. Kwon-Chung et al., 2014, S. C. Lee et al., 1996, J. R. Perfect et al., 1998, A. Sudan et al., 2013). Patients have a variety of symptoms including headache, fever, malaise, altered mental status and, less frequently, a depressed level of consciousness. Furthermore, elevated intracranial pressure is a common complication, and this condition can result in loss of vision and hearing (T. Bicanic and T. S. Harrison, 2004).

1.1.3 The burden of cryptococcal infection and HIV

As mentioned above, the worldwide burden of cryptococcosis is quite high with an estimate 1 million cases per annum in HIV/AIDS patients (B. J. Park, et al., 2009). The case fatality rate in patients with cryptococcal meningoencephalitis is between 35%-65% in sub-Saharan Africa (R. J. Lessells et al., 2011). This compares with a mortality rate of 10%-20% in most developed countries. The high mortality is mainly due to a delay in presentation of the disease, which leads to diagnosis only when meningoencephalitis is advanced and treatment is less effective. The high fatality rate is also partly due the poor availability, the high cost and the toxicity of the first-line anti-fungal treatments such as amphotericin B, as well as the frequent
complication of elevated intracranial pressure mentioned above (R. Rajasingham et al., 2012). The high burden of cryptococcosis demonstrates an urgent need for earlier diagnostic approaches, and improved treatment of cryptococcal disease, particularly in resource-poor settings such as Sub-Saharan Africa (B. J. Park, et al., 2009).

1.1.4 Treatment

The patient’s underlying conditions, such as HIV status, or the occurrence of an organ transplant, are major determinants of the treatment guidelines for cryptococcal meningoencephalitis (J. R. Perfect et al., 2010). For instance, the primary therapy for an HIV-infected patient is intravenous (IV) amphotericin B (AmB) deoxycholate (Ambd) at 0.7 - 1.0 mg/kg plus flucytosine at 100mg/kg for 4 days, followed by 2 weeks of fluconazole at 6 mg/kg (J. R. Perfect, et al., 2010, M. S. Saag et al., 2000, C. M. van der Horst et al., 1997). In some cases, a combination of antifungal drugs is used with immunomodulating agents such as monoclonal antibodies and gamma interferon as primary treatments (R. A. Larsen et al., 2005) (P. G. Pappas et al., 2004).

1.2 Virulence features of C. neoformans

A combination of fungal virulence factors and host susceptibility factors enable C. neoformans to survive and proliferate within vertebrates (A. Casadevall and L. A. Pirofski, 1999, J. N. Steenbergen et al., 2003). Several major virulence traits have been identified and each plays a unique role in the pathogenesis of disease caused by C. neoformans. These traits are described in the following sections.

1.2.1 Growth at 37°C

The ability to grow and proliferate at 37°C is an essential virulence attribute of pathogenic microorganisms that attack vertebrate hosts. Being an environmental yeast, a
key hallmark of *C. neoformans* is its ability to grow at the human body temperature of 37°C (J. A. Barnett, 2010, M. A. Garcia-Solache et al., 2013, L. R. Martinez et al., 2001, V. A. Robert and A. Casadevall, 2009). Although this trait is not traditionally considered to be a virulence factor, it has been proposed that any enzyme that confers fitness at high temperature would fit the definition of virulence factor (V. A. Robert and A. Casadevall, 2009). A number of proteins have been identified in *C. neoformans* that play a role in growth at high temperature. Some of the better characterized examples include Ras1, the p21-activated kinase (PAK) kinase Ste20, the phospholipid-binding protein Cts1, the vacuolar ATPase Vph1, the thiol peroxidase Tsa1, the cell integrity mitogen-activated protein (MAP) kinase Mpk1, and the Ca\textsuperscript{2+}/calmodulin-dependent phosphatase calcineurin (J. A. Alspaugh et al., 2000, T. Erickson et al., 2001, D. S. Fox et al., 2001, P. R. Kraus et al., 2003, T. A. Missall et al., 2004b, A. Odom et al., 1997, P. Wang et al., 2002). Deletion or disruption of a gene encoding one of these proteins results in either attenuation or complete loss of virulence in mammalian models of cryptococcosis (P. R. Kraus et al., 2004). A mouse model in which cryptococcal cells are introduced by inhalation is the most commonly used assay for differences in the virulence of strains and mutants.

### 1.2.2 Polysaccharide capsule

A prominent and characteristic feature of *C. neoformans* is the presence of a polysaccharide capsule that surrounds the cells. The capsule is visible by India ink staining as a halo where the ink is excluded from contact with the cell surface by the presence of the polysaccharide (Figure 1.1) (O. Zaragoza et al., 2009).
Figure 1.1: Microscopy image of India ink staining showing the characteristic halo caused by the presence of the polysaccharide capsule of *C. neoformans* strain H99

*C. neoformans* produces a complex polysaccharide made of glucuronoxylomannan (GXM) and galactoxylomannan (GalXM), and mannoproteins are also found associated with the capsule. The polysaccharides GXM and GalXM are attached to the cell surface as well as shed into culture medium; these polysaccharides can be isolated from culture supernatants and detected in the serum and cerebral spinal fluid of patients (M. Feldmesser and A. Casadevall, 1997, M. Feldmesser et al., 1996). Mannoproteins, minor component of the capsule, are heavily glycosylated that are shed during growth *in vivo* and *in vitro*. The functions of these proteins are poorly characterized with the exception that the mannoprotein Cig1 (the product of the *CIG1* gene) participates in iron acquisition from heme at the cell surface (B. Cadieux et al., 2013). Mannoproteins are highly immunogenic and can elicit protective immune responses in mice (M. K. Mansour et al., 2004, D. Pietrella et al., 2001). In general, the capsule polysaccharides have unique physical and chemical characteristics compared with capsules from bacterial pathogens, although the synthesis, transport and attachment of the *C. neoformans* material are poorly characterized.
It has been proposed that the capsule is antiphagocytic because it conceals cell-surface pathogen-associated molecular patterns, and capsule elaboration by cryptococcal cells leads to decrease in phagocytic uptake in the absence of opsonization (S. Shoham and S. M. Levitz, 2005). In addition to its antiphagocytic capacities, the capsule has many other proposed functions as a virulence factor. For example, it is proposed to protect *C. neoformans* from oxidative stress inside host cells by quenching reactive oxygen species (ROS) and other microbicidal molecules (P. K. Naslund et al., 1995, J. Rivera et al., 1998, O. Zaragoza, et al., 2009).

The contribution of capsule to virulence in mouse models has clearly been demonstrated because *C. neoformans* acapsular mutants are avirulent (Y. C. Chang and K. J. Kwon-Chung, 1994). However, one must consider and view every virulence factor in the context of the interaction with host factors. For example, the capsule is required for virulence in immunologically intact hosts, but acapsular strains can be pathogenic in mice lacking cell-mediated immunity (M. Del Poeta, 2004).

### 1.2.3 Melanin

*C. neoformans* secretes the copper-dependent enzyme laccase to the cell wall and this enzyme catalyzes the oxidative polymerization of phenolic catecholamines that are deposited as a dark pigment melanin in the cell wall (Figure 1.2) (I. Polacheck, 1991, P. R. Williamson, 1997).
Figure 1.2: The dark pigment melanin which is deposited in the cell wall in presence of laccase substrates such as L-3,4-dihydroxyphenylalanine (L-DOPA).

Melanin is a negatively charged hydrophobic pigment with antioxidant properties. It provides resistance to reactive oxygen and nitrogen species generated by host phagocytes and protects *C. neoformans* from oxidative killing by these immune effector cells (A. Casadevall et al., 2000). In particular, it has been experimentally demonstrated that melanized cells are resistant to killing both by oxidants (H. S. Emery et al., 1994) and by antifungal drugs such as AmB (D. van Duin et al., 2002). Furthermore, melanin production is influenced by changes in chromosome copy number, as *C. neoformans* strains that were monosomic for chromosome 13 produced more melanin and were more virulent than non-melanised strain disomic for chromosome 13 in a murine model of cryptococcosis (G. Hu et al., 2011).

### 1.2.4 Other virulence traits

In addition to the major virulence factors described earlier, many other proteins and enzymes are associated with virulence in *C. neoformans*. These are briefly described in the following sections.

#### 1.2.4.1 Urease

Urease appears to have an important role in promoting fungal traversal of the blood-
brain barrier to promote brain invasion (M. A. Olszewski et al., 2004, M. Shi et al., 2010, A. Singh et al., 2013). However, the mechanisms by which *C. neoformans* traverses tissue barriers, and the contribution of urease to these processes are not clearly established. In general, *C. neoformans* appears to move from the microvasculature of the brain into the CNS either via a “Trojan horse” mechanism involving macrophages or by direct transversal of free yeast cells (T. C. Sorrell et al., 2015, K. Vu, et al., 2014).

### 1.2.4.2 Phospholipase

Phospholipases degrade phospholipids. It has been shown that phospholipase B (Plb1) is secreted during *C. neoformans* infection and leads to an increase in capsule growth as well as enhanced fungal survival in phagocytes (G. M. Cox et al., 2001). One proposed mechanism for the contribution of Plb1 is that it degrades host phospholipids as part of a role in nutrient acquisition, perhaps by damaging phagosomal membranes and allowing fungal access to cytoplasmic components (C. J. Chrisman et al., 2011). Plb1 might also play a role in extrapulmonary dissemination because Plb1-deficient mutants have reduced virulence and decreased invasion of the brain (G. M. Cox, et al., 2001, R. Maruvada et al., 2012). A phospholipase C (Plc) was also recently shown to be involved in thermotolerance, capsule and cell wall synthesis, and virulence in *C. neoformans* (S. Lev et al., 2013).

### 1.2.4.3 Oxidative defenses

Host immune cells secrete reactive oxygen and nitrogen species (ROS and RNS). These molecules are potent producers of free radicals and are powerful microbicides. Inducible Nitric Oxide Synthase (iNOS) also contributes to the production of nitrogen free radicals species (E. Novo and M. Parola, 2008). It has been shown that chemically-derived
NO is fungicidal (J. A. Alspaugh and D. L. Granger, 1991) and in vivo NO is a major contributor to microbicidal mechanisms (D. Goldman et al., 1996, A. Vazquez-Torres et al., 2008).

*C. neoformans* employs numerous and redundant oxidative and nitrosative stress defense mechanisms, suggesting that this pathogen is well adapted to the oxidative defense environment in the host. Mouse infection studies revealed that *C. neoformans* mutants with defective nitrosative defenses are less virulent than the wild-type strain (T. A. Missall et al., 2004a). Additionally, oxidative stress-related genes, for example encoding superoxide dismutases (SOD) and catalases, are upregulated in *C. neoformans* upon uptake by macrophages (F. A. Brandao et al., 2015). *C. neoformans* possesses two SOD isoforms and it was shown that an SOD1-deficient mutant is more susceptible to macrophage killing and is slightly attenuated in mice (G. M. Cox et al., 2003). However, the SOD2 isoform is vital for aerobic growth at 37°C, and sod2 mutants are avirulent (S. S. Giles et al., 2005). Additional antioxidative enzymes in *C. neoformans*, such as thiol peroxidases, defend against both oxidative and nitrosative stresses and play a major role in cryptococcal virulence (T. A. Missall, et al., 2004a, T. A. Missall, et al., 2004b).

### 1.2.4.4 Secreted vesicles

*C. neoformans* produces vesicles that can cross the cell wall through an as yet unknown mechanism to reach the extracellular environment (M. L. Rodrigues et al., 2008a, M. L. Rodrigues et al., 2007, A. Yoneda and T. L. Doering, 2006). The vesicles are known to contain and traffic capsular material across the cell wall via exocytosis (M. L. Rodrigues et al., 2008b). In addition to capsular materials, vesicles carry most of the virulence factors mentioned earlier such as laccase, urease, and superoxide dismutase (J. M. Wolf et al.,
It has been suggested that *C. neoformans* may use the vesicles as part of an all-purpose secretion mechanism for trans-cell wall transport and delivery of virulence cargo to the extracellular environment (M. L. Rodrigues, et al., 2008a). Furthermore, it’s been shown that vesicles can contribute to the pathogenesis of cryptococcal meningoencephalitis and cryptococcal progression in central nervous system by facilitating traversal across the Blood Brain Barrier (BBB) (K. Vu et al., 2009). It has also been shown that a Sec6-dependent pathway plays a major role in release of these so-called “virulence delivery bags” because *sec6* RNAi mutants of *C. neoformans* are defective in a number of virulence factors including laccase, urease, capsule and have attenuated virulence in mice (J. Panepinto et al., 2009).

The exact mechanism by which the extracellular vesicles are produced, and how they cross the cell wall is largely unknown, but it has been suggested that they are potentially formed by a similar mechanism previously described for exosomes (C. V. Harding et al., 2013, R. M. Johnstone, 2005, J. D. Nosanchuk et al., 2008, M. L. Rodrigues, et al., 2008a). Briefly, it is suggested that *C. neoformans* multivesicular bodies fuse with the plasma membrane and release the extracellular vesicles; alternatively virulence factors may be secreted directly via exocytosis (T. L. Doering, 2009, J. D. Nosanchuk, et al., 2008, M. L. Rodrigues, et al., 2008a, A. Yoneda and T. L. Doering, 2009).

**1.2.4.5 Nutrient acquisition**

The intracellular and extracellular growth of *C. neoformans* in the host and the biosynthesis of major virulence factors including melanin and capsule require fungal access to nutrients. Among these nutrients, metals such as copper and iron are of great
importance for *C. neoformans* pathogenesis, and so the ability to acquire these minerals under conditions of limited availability in the host is essential for cryptococcal survival and pathogenicity. The following sections summarize the mechanisms of acquisition for iron and copper, and the relevance of these metals to cryptococcal virulence.

### 1.2.4.5.1 Iron

Both vertebrate hosts and microbial pathogens require iron as an essential element. Iron serves as a cofactor or a prosthetic group for essential enzymes in many basic cellular functions and metabolic pathways (J. Barasch and K. Mori, 2004, Y. Fu et al., 2004, M. Reyes and G. Imperatore, 2001, U. E. Schaible and S. H. Kaufmann, 2004, E. D. Weinberg, 1999). These functions and pathways include the synthesis of DNA as well as central energy metabolism, and iron is a critical co-factor in various proteins that serve as oxygen carriers and electron transfer systems. Ferric iron (Fe\(^{3+}\)) is almost insoluble under aerobic conditions, and free iron catalyzes the formation of oxygen radicals through the Fenton reaction (Figure. 1.3). These radicals are highly potent and damage cellular component including DNA, lipids, proteins and nucleic acids (J. A. Imlay et al., 1988).

![Figure 1.3](image)

**Figure 1.3:** The Fenton reaction. Reactive oxygen species (ROS) are generated in the Fenton reaction in the presence of iron and oxygen

In the host, iron is usually only available when it is bound to specific proteins such as transferrin (TF), lactoferrin (LF) and ferritin, or when it is complexed to heme within
hemoproteins (M. W. Hentze et al., 2004, U. E. Schaible and S. H. Kaufmann, 2004). The host deprives microbial pathogens of essential iron ions via a process called nutritional immunity (M. Nairz et al., 2014, N. L. Parrow et al., 2013, E. D. Weinberg, 2009). Iron availability in mammalian host fluids is maintained at extremely low levels \(10^{-18} \text{ M}\) (J. Hegenauer and P. Saltman, 1975, C. Wandersman and P. Delepelaire, 2004, E. D. Weinberg, 1974) and pathogenic microbes require \(10^{-6}\) to \(10^{-7} \text{ M}\) iron for growth in mammalian hosts (G. J. Anderson et al., 1994, G. J. Anderson and C. D. Vulpe, 2009, U. E. Schaible and S. H. Kaufmann, 2004). Therefore, pathogens have developed effective strategies to acquire iron from the limiting environment of the host. However, cellular iron levels are tightly regulated to avoid toxicity due to the formation of ROS. In general, iron homeostasis is considered as the balance between maintaining appropriate iron levels for cellular functions yet keeping levels sufficiently low or regulated to avoid iron-loading toxicity. Both host and pathogen cells have developed sophisticated systems for assuring a balanced cellular iron homeostasis (G. J. Anderson, et al., 1994, G. J. Anderson and C. D. Vulpe, 2009, Kaplan et al., 2006, S. Labbe et al., 2013, U. E. Schaible and S. H. Kaufmann, 2004). Specialized iron-acquiring systems in microbial pathogens consist of different molecules, which function in a highly interconnected fashion; these molecules include iron-binding small molecules called siderophores, the cognate siderophore uptake functions and high affinity ferric iron uptake systems (S. C. Andrews et al., 2003, P. Canessa and L. F. Larrondo, 2013, M. Caza and J. W. Kronstad, 2013, W. H. Jung and J. W. Kronstad, 2011, J. W. Kronstad et al., 2013, T. Lian et al., 2005).

A thorough understanding of the mechanisms for regulating iron uptake and homeostasis is important for understanding the competition between host and pathogen,
and could potentially identify new therapeutic targets for the treatment. Although much is known about iron acquisition by bacterial pathogens, much less is known about fungal pathogens. In this regard, *Saccharomyces cerevisiae* (baker’s yeast) and *Schizosaccharomyces pombe* (fission yeast) have been valuable fungal models for studying molecular and cellular mechanisms of iron acquisition. Yeasts are one of the simplest eukaryotes with deciphered genomes, and they are also simple to manipulate genetically; they have also facilitated the identification of proteins required for iron uptake, intracellular iron trafficking, and Fe-S cluster biogenesis in *C. neoformans* and other eukaryotes (J. De Freitas et al., 2003, A. C. Dlouhy and C. E. Outten, 2013, J. Encinar del Dedo et al., 2015, P. A. Frey and C. E. Outten, 2011, A. Kumanovics et al., 2008, S. Labbe, 2010, S. Labbe et al., 2007, H. Li and C. E. Outten, 2012, A. Mercier and S. Labbe, 2010, C. E. Outten and A. N. Albetel, 2013, B. Pelletier et al., 2003, C. B. Poor et al., 2014, M. Schrettl et al., 2004). The key features of iron homeostasis for *S. cerevisiae* and *S. pombe* are presented below to provide context for the subsequent description of related functions in *C. neoformans*.

### 1.3. Iron homeostasis in eukaryotic models: *S. cerevisiae* and *S. pombe*

#### 1.3.1 Iron homeostasis in *S. cerevisiae*

Iron homeostasis in *S. cerevisiae* is mediated by the expression of iron uptake and storage functions that are regulated by the iron-responsive transcription factor Aft1. Specifically, Aft1 regulates the transcription of a wide range of iron-responsive genes in yeast, termed the iron regulon, by repressing expression in the presence of high available iron. The iron regulon includes genes encoding components of the iron transport systems on the plasma membrane and the vacuolar membrane, that is, *ARN1-4, FRE1, FRE2, FET3,*
FTR1, FET5, FTH1 and SMF3 (C. Casas et al., 1997, R. F. Hassett et al., 1998a, R. F. Hassett et al., 1998b, A. Kumanovics, et al., 2008, R. Stearman et al., 1996, Y. Yamaguchi-Iwai et al., 1995, Y. Yamaguchi-Iwai et al., 1996, Y. Yamaguchi-Iwai et al., 2002). The *S. cerevisiae* genome also encodes a paralogue of Aft1, namely Aft2, which is 39% identical to Aft1 in the N-terminal DNA-binding domain (Kaplan et al., 2006). Both transcription factors activate gene expression in iron-deficient conditions, presumably via a similar mechanism. The sets of genes controlled by Aft1 and Aft2 are partially overlapping, and the role of Aft2 seems to be more significant in the absence of Aft1 (P. L. Blaiseau et al., 2001, J. C. Rutherford et al., 2001). The Aft1 and Aft2 proteins are located in the cytosol under iron-replete conditions and move to the nucleus under iron-depleted conditions, where they activate the expression of the Fe-responsive genes (M. Jbel et al., 2011, A. Mercier and S. Labbe, 2009). In addition to regulation at transcriptional level by Aft1/Aft1, iron homeostasis in yeast is also controlled by posttranscriptional mechanisms. Within the iron regulon mentioned earlier, there are genes encoding two mRNA-binding (mRNA-destabilizing) proteins, known as Cth1 and Cth2. These proteins are characterized by the presence of two zinc fingers (ZF). Cth1 is expressed in early stages of iron deficiency, whereas Cth2 is expressed during the persistence of iron limitation. Using their ZFs, Cth1 and Cth2 bind specific AU-rich elements (AREs) in the 3′UTRs of mRNAs and modulate the 3′-end processing of mRNAs to promote the degradation of target transcripts. Cth1 promotes the degradation of mRNAs encoding proteins that function in highly Fe-dependent processes, such as mitochondrial respiration, whereas Cth2 has a broader set of targets including functions of the tricarboxylic acid (TCA) cycle and respiration, lipid metabolism, heme synthesis, and amino acid biosynthesis (M. Martinez-Pastor et al., 2013,
S. Puig et al., 2008).

In addition to transcriptional and post-transcriptional levels mentioned above, iron homeostasis in yeast is further controlled at a post-translation level. It has been shown that the transcriptional activity of Aft1 is further regulated by and requires the two yeast cytosolic monothiol glutaredoxins (Grxs), ScGrx3 and ScGrx4. ScGrx3 and ScGrx4 maintain mitochondrial Fe–S cluster biosynthesis via the aminopeptidase P-like proteins Fra1 (Fe-repressor of activation-1) and Fra2 (Fe-repressor of activation-2 (A. Kumanovics, et al., 2008, L. Ojeda et al., 2006, N. Pujol-Carrion et al., 2006, J. C. Rutherford et al., 2005).

1.3.2 Iron homeostasis in S. pombe

To achieve iron homeostasis in S. pombe, iron-responsive gene expression is controlled by two repressor-type regulators Fep1 and Php4. Specifically, Fep1 and Php4 are responsible for controlling iron acquisition and iron utilization, respectively (Kaplan et al., 2006, S. Labbe, et al., 2007, C. H. Lillig et al., 2008, P. A. Lindahl and G. P. Holmes-Hampton, 2011). Fep1 is a GATA-type transcription factor that binds to GATA-containing sequences in promoters of iron uptake and transport genes under iron-replete conditions, repressing their expression to avoid iron overload. Fep1 orthologs are well conserved across various fungal species.

Php4 is another arm of the maintenance system for iron homeostasis in S. pombe. Php4 is a transcriptional repressor and regulates expression of genes in iron-dependent metabolic pathways and forms a protein complex with Php2, Php3, and Php5 (M. G. Khan et al., 2014, A. Mercier and S. Labbe, 2009, A. Mercier et al., 2006, A. Mercier et al., 2008, P. Vachon et al., 2012). With sufficient levels of iron in the cell (i.e., iron-replete conditions) Php4 is not expressed and the Php2/Php3/Php5 complex activates expression of its target
genes by binding to CCAAT sequences in their promoters. These target genes encode proteins involved in iron-sulfur cluster biogenesis, heme biosynthesis, the mitochondrial electron transport chain, and the TCA cycle. Under low-iron conditions, Php4 is expressed and binds to the Php2/Php3/Php5 complex, resulting in a switch of the complex from an activator to a repressor. This leads to down regulation of iron-utilizing pathways as an iron-sparing response to low levels of iron under iron-depleted condition (A. Mercier and S. Labbe, 2009, A. Mercier, et al., 2006, A. Mercier, et al., 2008).

1.4 Monothiol glutaredoxins

Monothiol Grxs with active-site cysteine-glycine-phenylalanine-serine (CGFS) amino acid motifs are a family of proteins that are evolutionarily conserved and found in organisms ranging from bacteria to humans. Monothiol Grxs possess specific structural domains as shown in Figure 1.4.
Figure 1.4: Comparison of domain structures across species for monothiol glutaredoxins. The positions and sizes of glutaredoxin (red) and thioredoxin-like (green) domains are indicated. Numbers represent the position of the cysteine residue in the active site CGFS motif, and the total lengths of the proteins (Adapted from Outten et al Biochemistry, 2012, 51 (22), pp 4377–4389).

In general, this family of Grx proteins is divided into two subclasses. The first subclass includes those with a single Grx domain (e.g., E.coli (Ec) Grx4). The second subclass includes multi-domain proteins comprised of an N-terminal thioredoxin-like domain as well as one (Sc, Grx3 and Grx4), two (Hs, Grx3) or three (At, Grx3) Grx domains.
Yeast Grx3 and Grx4 are highly homologous and are thought to possess redundant functions in the cell. A grx3Δ grx4Δ double mutant results in constitutive expression of the iron regulon and shows a significant growth defect (L. Ojeda, et al., 2006, N. Pujol-Carrion, et al., 2006). All bacterial monothiol CGFS Grxs are of the single Grx domain type, while in eukaryotes, both the single Grx domain and the multi-domain Grxs are present (J. Couturier et al., 2009, E. Herrero and M. A. de la Torre-Ruiz, 2007, F. Vilella et al., 2004). In addition to the three monothiol Grxs mentioned above, S. cerevisiae also possesses two other monothiol Grxs, ScGrx6 and ScGrx7. ScGrx6 is the first monothiol Grx shown to directly bind an iron-sulfur cluster that is stabilized by reduced glutathione (N. Mesecke et al., 2008).

1.4.1 Roles of monothiol Grxs

Traditionally, Grxs function in the reduction of protein disulfides or the glutathionylation of proteins (J. Couturier, et al., 2009, E. Herrero and M. A. de la Torre-Ruiz, 2007, C. H. Lillig, et al., 2008). However, recent biochemical characterization and in vivo studies indicate that monothiol CGFS Grxs have conserved roles in iron metabolism in multiple organisms, either through a role in the iron-sulphur cluster (ISC) assembly machinery or through the regulation of iron-responsive transcription factors (F. Vilella, et al., 2004) (S. Bandyopadhyay et al., 2008a, Kaplan et al., 2006, H. Li and C. E. Outten, 2012, R. Lill et al., 2012, A. Mercier and S. Labbe, 2009, U. Muhlenhoff et al., 2010, N. Rouhier et al., 2010).

1.4.1.1 Role of monothiol Grx3/4 in regulation of Aft1 in yeast

As mentioned above, under iron deficient conditions, Aft1 accumulates in the nucleus, and under iron-replete conditions, Aft1 is shuttled to the cytosol by the nuclear
exportin Msn5 (R. Ueta et al., 2007, Y. Yamaguchi-Iwai, et al., 1995, Y. Yamaguchi-Iwai, et al., 2002). It is known that Fe-S-ligated Grx3/4 promotes dissociation of Aft1 from its DNA target and Grx3/4 proteins are required to control the activity of Aft1 (R. Ueta et al., 2012). Briefly, Grx3 and Grx4 form [2Fe-2S] cluster-binding complexes that, together with the cytosolic proteins Fra1 and Fra2, transmit an inhibitory signal to Aft1 that is dependent on the presence of glutathione and the synthesis of mitochondrial Fe-S clusters (A. Kumanovics, et al., 2008, H. Li et al., 2011, H. Li et al., 2009, H. Li and C. E. Outten, 2012, U. Muhlenhoff, et al., 2010, J. C. Rutherford, et al., 2005). Moreover, it has been shown that GSH depletion in S. cerevisiae leads to manifestation of similar phenotypes to those associated with loss of iron-dependent inhibition of Aft1/Aft2 (J. C. Rutherford, et al., 2005, K. Sipos et al., 2002).

1.4.1.2 Role of monothiol Grx4 in the regulation of Fep1 in fission yeast

The fission yeast S. pombe contains two CGFS-type monothiol glutaredoxins, Grx4 and Grx5, which are localized in the nucleus and mitochondria, respectively. Grx4 is required for iron limitation-dependent inhibition of Fep1 (M. Jbel, et al., 2011, K. D. Kim et al., 2011). Lack of Grx4 caused defects not only in growth but also in the expression of both iron-uptake and iron-utilizing genes regardless of iron availability. In order to identify the mechanisms by which Grx4 is involved in Fep1-mediated regulation, the protein-protein interactions for these proteins were investigated. Co-immunoprecipitation and bimolecular fluorescence complementation (BiFC) revealed that Grx4 physically interacts with Fep1 in vivo. BiFC identified punctate signals in the nucleus that were produced by the interaction of Grx4 with Fep1. Based on comparisons with S. cerevisiae, it has been suggested that Grx4 most likely binds Fe-S cofactors and could be critical in
interacting with Fep1 to modulate its activity.

1.4.1.3 Role of monothiol Grx4 in regulation of Php4 in fission yeast

Monothiol Grx4 regulates the activity of Php4 in *S. pombe* through specific protein–protein interactions. Yeast two-hybrid and bimolecular fluorescence complementation experiments have shown that Grx4 physically interacts with Php4 regardless of cellular iron levels, and under iron-replete conditions, Grx4 promotes the export of Php4 to the cytosol. It has been shown that deletion of *GRX4* leads to constitutive nuclear localization of Php4 and constitutive repression of Php4-regulated genes (M. A. Huynen et al., 2005, M. Jbel, et al., 2011, K. D. Kim, et al., 2011). Similar to scenarios discussed above for Aft1 and Fep1, it has been shown that Fe-S binding via multidomain CGFS Grx4 is an important factor required for inhibition of Php4, as mutation of the CGFS motif in Grx4 disrupts Fe-dependent inhibition of Php4. In addition, and similar to *S. cerevisiae*, depletion of glutathione leads to iron-dependent inhibition of Php4, most probably stemming from the requirement for GSH as a [2Fe-2S] cluster ligand in CGFS Grx homodimers (H. Li and C. E. Outten, 2012, R. Lill, et al., 2012).

1.4.1.4 Monothiol CGFS Grxs contain a labile Fe–S cluster

Fe-S clusters are cofactors that play important roles in cellular functions such as redox reactions, electron transport, and regulatory reactions. The synthesis of Fe-S clusters occurs exclusively at the mitochondrial matrix, independently of whether the final destination of the Fe-S clusters is to mitochondria, nuclear or cytosolic proteins. After synthesis, the clusters can be exported from the mitochondria, and additional iron-sulphur cluster assembly proteins (CIA machinery) are required to assemble Fe-S clusters into apoproteins in the cytosol or the nucleus (R. Lill et al., 2006, R. Lill and U. Muhlenhoff,
Recent studies indicate that the recombinant versions of bacterial, human, yeast and plant monothiol Grxs (produced in *E. coli*) are able to incorporate labile Fe–S clusters (S. Bandyopadhyay et al., 2008b, M. M. Molina-Navarro et al., 2006, B. Zhang et al., 2013). Analytical and spectroscopic analyses of recombinant purified Grxs under anaerobic conditions demonstrated the incorporation of labile \([\text{Fe}_2\text{S}_2]^{2+}\) clusters in the presence of glutathione (GSH) (S. Bandyopadhyay, et al., 2008b, T. Iwema et al., 2009, A. Picciocchi et al., 2007, N. Rouhier et al., 2007).

As introduced above, the role of Grx3/4 in the regulation of Aft1 in yeast is mediated by the ability of monothiol Grxs to provide a bridging and glutathione-containing Fe/S center, that functions both as an iron sensor and in intracellular iron delivery (U. Muhlenhoff, et al., 2010, L. Ojeda, et al., 2006). It has also been shown that GSH depletion in yeast leads to constitutive activation of Aft1 and drastic intracellular iron accumulation which causes toxicity and cell death (J. C. Rutherford, et al., 2005).

1.5 Glutathione

The tripeptide, Y-L-Glutamyl-L-cysteiny1-glycine, or glutathione (GSH) is the major low-molecular-weight thiol compound found in almost all organisms including plants, animals and most pathogens (A. Meister, 1984). GSH is synthesized from L-glutamate, L-cysteine, and glycine in two steps, catalyzed by Y-glutamyl-cysteine synthase and glutathione synthase (S. C. Lu, 2009). The peptidic Y-linkage is known to protect GSH tripeptide against degradation by aminopeptidases. In addition to the reduced form, GSH is present in several other forms. Glutathione disulfide (GSSG) is formed upon oxidation, which is often referred to as “oxidized glutathione,” or “glutathione disulphide”. Other
forms of disulfide are of the mixed type, for instance GSSR, is a major class of glutathione-cysteinyldisulfides on proteins. This form of protein is referred to as “glutathionylated” or “thiolated” (S. M. Beer et al., 2004, K. P. Huang and F. L. Huang, 2002, C. Luchese and C. W. Nogueira, 2010, Z. D. Zhou and T. M. Lim, 2010).

1.5.1 Roles of glutathione

GSH has a high cellular abundance (3 - 10 mM) and a low redox potential of −240 mV. These properties give GSH the special attribute of serving as a redox buffer that is important for reducing oxidized cysteine residues and also for protecting them from irreversible oxidation (F. Q. Schafer and G. R. Buettner, 2001). All major cellular functions involve the thiol redox state (D. Trachootham et al., 2008). GSH also participates in scavenging various endogenous and exogenous electrophilic compounds (C. M. Grant et al., 1999, A. Meister and M. E. Anderson, 1983, M. D. Shelton and J. J. Mieyal, 2008).

Since the discovery of GSH in 1889, the focus on GSH has increased in the last three decades and numerous functions of GSH have been elucidated (N. S. Kosower and E. M. Kosower, 1978). These include its biochemical properties, (H. F. Edelhauser et al., 1976, F. J. Giblin et al., 1976, W. A. Gunzler et al., 1974, J. Yam et al., 1976), and its significance in medical and clinical aspects, including carcinogenesis and drug resistance (A. P. Fernandes and A. Holmgren, 2004, J. D. Hayes and C. R. Wolf, 1990, S. Orrenius et al., 1983, B. Pool-Zobel et al., 2005, J. Vina et al., 1995a, J. Vina et al., 1995b).

research, which is also developing at a fast pace, is “protein glutathionylation” which has been suggested to have a major role in control of the functions mentioned earlier. For instance, phosphorylase, carbonic anhydrase, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and other proteins have been shown to undergo glutathionylation (Y. C. Chai et al., 1991, R. M. Miller et al., 1990, J. A. Thomas et al., 1994). It has also been shown that Human immunodeficiency virus (HIV)-1 protease activity is regulated by reversible glutathionylation (D. A. Davis et al., 1996), and that GSH levels determines the outcome of the disease in HIV patients (L. A. Herzenberg et al., 1997, J. D. Peterson et al., 1998).

1.5.1.1 A vital function of glutathione in iron homeostasis

One recent research area for GSH, which has expanded rapidly in the last 10 years, and is also challenging the traditional view of “GSH” as the main player in thiol-redox control, is the critical role of GSH in iron homeostasis (C. Kumar et al., 2011). It has been shown that GSH serves as a point of intersection between iron metabolism and thiol-redox maintenance, and partners with monothiol Grxs to assemble Fe–S clusters (H. Li, et al., 2011, H. Li, et al., 2009, H. Li and C. E. Outten, 2012, D. T. Mapolelo et al., 2013). GSH is also a major component of the labile iron pool (LIP) of available iron in cells, and this adds significance to the role of GSH in iron homeostasis (R. C. Hider and X. L. Kong, 2011). In this regard, GSH is a major cytosolic ligand for Fe (II). In eukaryotes, the majority of iron taken up by transporters and iron acquisition components enters a metabolically active pool in the cytosol known as the labile iron pool (LIP) (M. Shvartsman and Z. Ioav Cabantchik, 2012). The properties and features of this cytosolic pool are not completely understood, but the LIP is defined as the freely exchangeable iron that is loosely coordinated by water, small molecules, and proteins. Because the cytosolic LIP is
essentially present as Fe (II), the physiologic ligands for the cytosolic LIP must have affinity for this form of iron. Analysis of potential ligands in vitro suggests that Fe-GSH complexes are likely the only species that forms at significant levels in cells, mainly due to the fact that GSH is present at relatively high levels in the cytosol and has moderate affinity (Kd~8 μM) for Fe (II) (R. C. Hider and X. L. Kong, 2011, D. Kaur et al., 2009, C. Kumar, et al., 2011, R. Kumar, 1979).

1.5.1.1.1 Role of glutathione in iron homeostasis in fungi

As mentioned in sections 1.4 and 1.5.1.1, the role of cytosolic monothiol glutaredoxins Grx3 and Grx4 in intracellular iron trafficking and sensing in yeast is well established and its been shown that depletion of Grx3/4 specifically impairs all iron-requiring reactions in the cytosol, mitochondria, and nucleus, including the synthesis of Fe/S clusters and heme (U. Muhlenhoff, et al., 2010, Li et al., 2012). It has also been shown that GSH is required for maturation of cytosolic Fe-S clusters. (H. Li et al., 2012, K. Sipos, et al., 2002, U. Muhlenhoff, et al., 2010). Studies by Muhlenhoff et al., 2010 uncovered an important role of GSH in partnership with Grx3/4 in cellular iron metabolism in yeast. They showed that a crucial task of Grx3/4 is mediated by a bridging glutathione-containing Fe/S center that functions both as an iron sensor and in intracellular iron delivery. In fungi, recent studies have provided additional clues about the role of GSH (Rutherford et al., 2005 Mercier et al., 2008). It is known that the S. cerevisiae transcription factor Aft1 is activated by iron deficiency and inactivated by iron repletion, and it has been shown that the presence of GSH is required for iron-mediated inactivation of Aft1 function (J. C. Rutherford, et al., 2005). In fact, in GSH-deficient cells, the S. cerevisiae iron regulon genes are constitutively upregulated and are less responsive to iron
repression (J. C. Rutherford, et al., 2005). Like Aft1, *S. pombe* Php4 is active during iron deficiency, except that it represses transcription.

The significance of GSH in regulation of iron homeostasis is further underscored by the fact that Aft1 and Php4 are evolutionarily distant, but the presence of GSH is needed for iron-dependent inactivation of them both (W. H. Chung et al., 2005). As mentioned earlier, GSH is synthesized in two steps by Y-glutamylcysteine synthetase (GCS) and glutathione synthetase. Null mutations of the genes encoding GCS in *S. cerevisiae* (*GSH1*) and *S. pombe* (*gcs1*) lead to GSH auxotrophy, indicating an essential requirement of GSH in these yeasts (B. Chaudhuri et al., 1997, C. M. Grant et al., 1996, D. Wu et al., 1994). It has also been shown that Y-glutamylcysteine synthetase (GCS) is essential in *Candida albicans*, as the *gcs1/gcs1* null mutants in this fungal pathogen also exhibit glutathione auxotrophy, which could be rescued by supplementing with reduced and oxidized glutathione and Y–glutamylcysteine, indicating that glutathione biosynthesis (and glutathione itself) is essential *C. albicans* (Y. U. Baek et al., 2004).

Additionally, dimorphic zoopathogenic fungi produce and secrete a family of iron reductases that use GSH as ligand and this complex is involved in the acquisition and utilization of iron. Briefly, it was shown that culture supernatants from *Blastomyces dermatitidis*, *Histoplasma capsulatum*, *Paracoccidioides brasiliensis* and *Sporothrix schenckii* strains grown in their yeast forms were able to reduce iron enzymatically, with GSH as a cofactor (R. Zarnowski and J. P. Woods, 2005)

1.6 **Iron uptake in *C. neoformans***

Similar to other fungi, including the model yeasts, filamentous fungi, and fungal pathogens, *C. neoformans* employs various strategies to acquire iron from the environment
or from the host. These strategies include cell surface reductases that reduce ferric iron to its ferrous state, a high-affinity iron uptake pathway, a low-affinity iron uptake pathway, the use of siderophore-bound iron and siderophore transporters, and the acquisition of iron from heme. *C. neoformans* also exports reductants such as 3-hydroxyanthranilic acid (Figure 1.5).

### 1.6.1 The high-affinity iron uptake pathway

The high-affinity ferroxidase (Fet3)/permease (Ftr1) complex has been characterized in several fungi, including *S. cerevisiae* and *C. albicans* (R. Stearman, et al., 1996), (R. S. Almeida et al., 2008, J. Kaplan et al., 2006, S. A. Knight et al., 2005, D. J. Kosman, 2003, N. Ramanan and Y. Wang, 2000). Briefly, ferrous iron is first oxidized by the ferroxidase Fet3 before transport into the cytosol as ferric ion by the permease Ftr1. In *C. neoformans*, the Fet3 and Ftr1 orthologs Cfo1 and Cft1 are essential for ferric iron uptake, iron acquisition from transferrin, and full virulence in mice (W. H. Jung et al., 2008, W. H. Jung et al., 2009).

It has also been shown that the iron permease/ferroxidase complex is induced by low levels of iron (T. Lian, et al., 2005) and is localized at the plasma membrane (E. S. Jacobson and J. D. Hong, 1997, K. J. Nyhus et al., 1997) (W. H. Jung, et al., 2008) (W. H. Jung, et al., 2009).

### 1.6.2 Low-affinity iron uptake pathway

As mentioned above, iron uptake relies on the high-affinity uptake system in conditions of low iron concentration while low-affinity uptake systems are used when substrate is present in high concentrations. A low-affinity iron uptake pathway has been described in *S. cerevisiae* (C. C. Philpott, 2006, R. Sutak et al., 2008). Fet4 is described as a
non-ATP-dependent trans-membrane transporter, and the main component of the pathway (R. M. Dixon et al., 1994). The permease component of the low-affinity iron uptake are not specific to iron and take up other metals such as copper and zinc (Kaplan et al., 2006). Similar to *S. cerevisiae* in the presence of sufficient levels of iron, iron uptake is mediated by the low-affinity iron uptake pathway in *C. neoformans* (E. S. Jacobson et al., 1998). This system employs reductants, such as melanin, 3-hydroxyanthranilic acid (3HAA), and transporters. The exact mechanism of the iron uptake pathway is not fully known (Jacobson and Hong, 1997).

1.6.3 **Siderophore-bound iron transport**

The siderophore non-reductive iron uptake pathway was first described in *S. cerevisiae* and several fungal species (C. C. Philpott, 2006) (P. Heymann et al., 2002) (E. Lesuisse et al., 2002). The siderophore-iron complexes are transported into the cell via siderophore transporters (Sit). *S. cerevisiae* possesses four siderophore transporters and each transports a specific kind of siderophore (D. J. Kosman, 2003). The mechanisms of iron acquisition are generally conserved among fungal pathogens although there is variability in the dependence on siderophores and the ability to use heme as an iron source (R. S. Almeida et al., 2009, H. Haas, 2012, D. Kornitzer, 2009). *C. neoformans* does not produce siderophores but it does possess several predicted siderophore transporters. Siderophore transporter 1 (Sit1) has been characterized as the specific transporter for the siderophore ferrioxamine B (K. L. Tangen et al., 2007). Loss of Sit1 results in poor growth under iron-limiting conditions and increased melanin production; however, Sit1 is not required for virulence in a mouse model of cryptococcosis (K. L. Tangen, et al., 2007).
1.6.4  Heme uptake

Pathogenic fungi utilize heme as an iron source (R. Santos et al., 2003) (L. A. Foster, 2002). The majority of iron in human body is present in heme and so this source may be particularly important for *C. neoformans*. It has been shown that *C. neoformans* can also utilize iron from heme (Jung et al., 2008), and that the ESCRT protein Vps23 plays a role in heme acquisition. Deletion of *VPS23* resulted in a growth defect on heme, a capsule defect and attenuation of virulence in a mouse model (G. Hu et al., 2013). The mannoprotein Cig1 also plays an important role in iron acquisition from heme in *C. neoformans*. The transcript for the extracellular mannoprotein Cig1 is highly unregulated in low levels of iron and Cig1 is required for growth on heme, suggesting that Cig1 may function as a hemophore at the cell surface, suggesting that Cig1 may therefore function as a hemophore at the cell surface. Cig1 contributed to virulence in a mouse model of cryptococcosis but only in a mutant that also lacked the high-affinity iron uptake system B. Cadieux, et al. (2013).

1.6.5  Cir1: Master regulator of iron

The GATA-type transcription factor Cryptococcus Iron Regulator 1 (Cir1) is a key regulator of the transcriptional response to iron in *C. neoformans*. Cir1 was identified based on sequence similarity to the iron regulators in other fungi (W. H. Jung et al., 2006). Urbs1 from *Ustilago maydis*, SRE in *Neurospora crassa*, and Fep1 in *S. pombe* had been identified and the list of related proteins now includes Sfu1 in *C. albicans*, SREB in *H. capsulatum*, and Sre1 in *B. dermatitidis* (Z. An et al., 1997b, L. Y. Chao et al., 2008, G. M. Gauthier et al., 2010, K. A. Harrison and G. A. Marzluf, 2002, C. Y. Lan et al., 2004, B. Pelletier et al., 2002). These transcription factors possess a conserved cysteine-rich region (CRR) flanked by two zinc finger motifs characteristic of GATA-type transcription factors.
in higher eukaryotes. For instance Fep1 and Urbs1 bind 5’-GATA-3’ sequences in the promoters of genes encoding high-affinity iron uptake system as well as siderophore transport functions (Z. An et al., 1997a, B. Pelletier, et al., 2003). The CRR has been demonstrated to coordinate iron binding, affecting, for example, the DNA binding affinity of the SRE1 transcription factor of *H. capsulatum* (L. Y. Chao, et al., 2008) and in *S. pombe*; mutation of CRR cysteine residues of the Fep1 protein leads to increased mRNA levels of its target gene, the multicopper oxidase Fio1 (B. Pelletier et al., 2005).

The major impact of Cir1 was demonstrated by a comparison of the transcriptomes of the wild-type (WT) strain and the *cir1* mutant that revealed differential regulation by Cir1 of 2,311 genes under the iron-limited condition and 1,623 genes upon iron repletion (out of ~6500 genes in the genome). For example, Cir1 positively regulated genes for siderophore transport and negatively regulated genes for reductive iron uptake and melanin synthesis. Loss of Cir1 attenuated virulence and influenced the elaboration of all of the major virulence factors, including derepression of melanin production, and reduced capsule production and growth at 37°C (W. H. Jung and J. W. Kronstad, 2011, W. H. Jung, et al., 2006).

Overall, Cir1 regulates iron homeostasis and virulence factor expression (Jung et al., 2006). A recent study indicates that Cir1 also influences metabolic processes that require iron-dependent enzymes such as glycolysis, ergosterol biosynthesis, and inositol metabolism (J. N. Choi et al., 2012). It is not clear how many of these functions are directly regulated by Cir1 and which involve downstream transcriptional regulators. It has been suggested that there is potentially a crosstalk between these multiple processes and iron homeostasis.
**Figure 1.5: Components of iron network in C. neoformans.** High affinity uptake pathway that depends on ferric reductase activity, the iron permease Cft1 and the ferroxidase Cfo1 is highlighted in light blue, iron reductases (Fre1–8), and cell wall melanin (produced by the laccases Lac1 and Lac2) have ferric reducing activities along with (3-HAA). Six siderophore transporters are predicted from the genome, heme uptake system involves the mannoprotein Cig1, and ESCRT protein Vps23. Transcription factors such as Cir and HapX control the expression of these components. (Figure adapted from Kronstad et al. [http://dx.doi.org/10.1016/j.tim.2013.05.00])

### 1.7 Rationale and aims of study

As mentioned in section 1.6, iron acquisition and the maintenance of iron
homeostasis are critical for the pathogenesis of *C. neoformans*. The GATA-type, zinc-finger protein Cir1 controls the iron regulon and the known major virulence factors in *C. neoformans*. Thus understanding the mechanisms by which Cir1 senses iron availability and contributes to proliferation of the fungus in mammalian hosts is of great significance.

The studies reported in this thesis aimed to identify additional components of iron acquisition functions that are essential for *C. neoformans* growth to add depth to our understanding of the complex iron regulatory network. Importantly, the identification and characterization of components of the iron regulatory and uptake network in *C. neoformans* could serve as new targets for the design and development of more efficient and less toxic novel therapies for cryptococcosis.

### 1.7.1 Hypotheses

Initially, I embarked on a project in which potential binding partners of Cir1 were identified using a binding assay and a proteomic approach. This preliminary proteomic analysis of binding partners for Cir1 identified a monothiol glutaredoxin (Grx) encoded by the gene CNAG_02950). Based on the conserved evolutionary functions of monothiol Grxs in Fe-S cluster binding and regulation of iron homeostasis, as detailed in section 1.4, I hypothesized that Grx4 plays a role in iron homeostasis and contributes to the pathogenesis of *C. neoformans*. Importantly, a role for monothiol Grxs has not been explored previously in pathogenic fungi.

Furthermore, an important observation in Chapter 2 was that after 48 hours of iron starvation, a *grx4* mutant had a growth defect in the presence of iron compared to WT. I therefore tested the effect of exogenous GSH on the growth defect of *grx4* in the presence of iron after a period of iron starvation. Interestingly, the growth defect of the *grx4* mutant was rescued by exogenous GSH. As detailed in section 1.5 of this chapter, it is known that
glutathione protects against detrimental effects of iron-deficiency and iron toxicity due to its antioxidant properties. Also monothiol glutaredoxins require GSH to exert their function in the maintenance of iron homeostasis, and GSH is known as the major and essential component of iron binding ligand in cytosol and affect intracellular distribution of iron. These observations, in addition to the findings on the effect of GSH on the grx4 mutant outlined in Chapter 2, prompted me to hypothesize that glutathione influences iron homeostasis in C. neoformans. I examined the impact of deletion of the gene encoding glutathione synthetase in Chapter 3. The physiological effect of GSH has not been investigated in C. neoformans prior to my study.

1.7.2 Research objectives

The general objectives of my PhD studies were to elucidate the role of Grx4 and effect of GSH in iron homeostasis of C. neoformans. The main objective in Chapter 2 was to examine the role of the monothiol glutaredoxin Grx4 in the iron homeostasis and virulence in C. neoformans. The specific goals were to examine the Grx4-Cir1 interaction, analyze the impact of a GRX4 deletion on iron and virulence-related phenotypes and finally evaluate the impact of GRX4 on expression of known targets that regulate iron homeostasis in C. neoformans.

I continued the analysis on Grx4 by investigating transcript variants and the essentiality of the GRX4 locus. The specific goal of this follow-up study was to examine the transcript(s) structure of GRX4 locus with N-terminal variations by RNA-Seq analysis, and to test the essentiality of N-terminal region of GRX4 locus.

My objective in Chapter 3 was to investigate the physiological role of glutathione in C. neoformans. The specific goal of this section was initially the identification and
subsequent deletion of candidate targets in the GSH biosynthesis pathway in C. neoformans. Subsequently, I focused on the GSH2 gene encoding glutathione synthetase with a detailed analysis of phenotypic outcomes manifested upon deletion of GSH2.
Chapter 2: Monothiol glutaredoxin-4 is required for iron homeostasis and virulence in the AIDS-associated pathogen Cryptococcus neoformans

2.1 Introduction

Pathogenic microorganisms need to overcome nutritional barriers to proliferate in the hostile host environment (K. Fischer et al., 2004). For example, both vertebrate hosts and microbial pathogens require iron as an essential cofactor or a prosthetic group, either alone or bound to Fe-S clusters, in a plethora of cellular and metabolic functions. The dependence of both host and pathogen on iron leads to a competition for this essential nutrient. The host actively restricts the bioavailability of iron to the pathogen by iron-withholding mechanisms, which is countered by microbial strategies to effectively compete for iron during infection (S. Brill et al., 2000, M. Caza and J. W. Kronstad, 2013).

Iron is essential for cell viability as an important cofactor in a variety of enzymes that are linked to essential cellular functions including DNA synthesis, mitochondrial respiratory pathway and TCA cycle, oxidative phosphorylation, and the biosynthesis of metabolites. Ironically however, the same redox-active properties of this metal make it cytotoxic because iron undergoes Fenton-type chemical reactions that result in the production of hydroxyl radicals that can lead to cell death (M. W. Hentze et al., 2010, C. C. Philpott et al., 2012) (B. Halliwell and J. M. Gutteridge, 1992, J. A. Imlay, et al., 1988, J. A. Imlay and S. Linn, 1988). Therefore, cells have developed homeostatic mechanisms to acquire adequate, but not excessive, concentrations of iron. Optimal intracellular levels of iron are therefore critical for cell survival and organisms have developed tightly regulated mechanisms to sense and maintain iron homeostasis.

Competition for iron between microbes and mammalian hosts makes iron sensing
and acquisition key aspects of virulence for the pathogenic yeast *C. neoformans* (K. H. Bartlett, et al., 2008). As introduced in Chapter 1, *C. neoformans* causes invasive mycosis and severe meningoencephalitis in two immunocompromised risk groups: 1) individuals infected with the human immunodeficiency virus (HIV), in particular those with acquired immunodeficiency syndrome (AIDS), and; 2) organ transplant recipients (T. G. Mitchell and J. R. Perfect, 1995). The widespread use of highly active antiretroviral therapy (HAART) has decreased the incidence of cryptococcosis in developed countries (R. Manfredi et al., 1999, D. W. Mackenzie, 1989). However, the mortality rate and incidence of infection remains high in areas where access to HAART is limited. The burden of cryptococcal disease underlines the urgent need for new drug and vaccine targets including, potentially, functions for iron homeostasis.

Iron regulates the expression of the main virulence factors of *C. neoformans* including the production of a polysaccharide capsule and the deposition of melanin in the cell wall. *C. neoformans* is remarkably tuned to perceive iron as part of the disease process and employs various iron regulators and uptake mechanisms that contribute to virulence. These include high and low affinity iron uptake systems as well as heme uptake pathways (B. Cadieux, et al., 2013). High affinity uptake involves reduction of ferric iron (Fe$^{3+}$) to ferrous form (Fe$^{2+}$) by cell surface reductases, with subsequent transport by a permease (Cft1) and ferroxidase (Cfo1) (G. Hu, et al., 2013, E. S. Jacobson, et al., 1998, J. W. Kronstad et al., 2011). Its been shown that defects in iron acquisition attenuate virulence, and iron availability influences the expression of major virulence factors. Iron overload exacerbated manifestation of cryptococcal meningitis in a mouse model of infection and facilitated colonization of the brain (Jung *et al.*, 2008; 2009).
The GATA-type, zinc-finger protein Cir1 regulates iron uptake, iron homeostasis and virulence factor expression in *C. neoformans*. Mutants defective in *CIR1* exhibit iron-related phenotypes. Transcriptional profiling revealed that Cir1 regulates the majority of genes encoding iron uptake functions as well as genes for signal transduction, transcription, sterol biosynthesis, DNA replication, and cell wall biosynthesis (W. H. Jung, et al., 2006). However, the mechanisms by which Cir1 senses iron availability are important aspects of the proliferation of the fungus in the host is not entirely known. So far it has been shown that iron influences abundance of Cir1 and that levels of Cir1 protein decreases upon iron deprivation. This destabilization is also shown to be influenced by reducing conditions and by inhibition of proteasome function; In addition, a post-translational mechanism for the control of Cir1 abundance in response to iron and redox status has been suggested (W. H. Jung and J. W. Kronstad, 2011). Given the importance of iron homeostasis in *C. neoformans* pathogenesis, there is a clear need to identify further major components that contribute to Cir function and iron regulation to fully understand the disease process.

The mechanisms by which iron-responsive transcription factors in fungi sense intracellular iron levels and regulate iron homeostasis are best understood in *S. cerevisiae* and *S. pombe* (M. Jbel, et al., 2011). In these fungi, the transcription factors interact with monothiol glutaredoxins (Grxs) that participate in iron sensing and regulation. Recent studies in *S. cerevisiae* and *S. pombe* have demonstrated essential roles for monothiol Grxs in intracellular iron homeostasis, iron trafficking and the maturation of Fe-S cluster proteins, and have established the proteins as novel Fe-S cluster-binding regulatory partners. (M. Jbel, et al., 2011). In addition several studies point to critical roles for monothiol Grxs in the maintenance of cellular iron homeostasis (N. Rouhier, et al., 2010).
In this chapter, a proteomic approach was initially taken by Dr. Horacio Bach to identify potential binding partners of Cir1 that may contribute to the complex and dynamic iron-related regulatory network of \textit{C. neoformans}. The monothiol Grx4 was identified (gene ID: CNAG_02950) as a potential binding partner of Cir1 by mass-spectrometry. Given the conserved role of monothiol Grxs in iron homeostasis, I hypothesized that the monothiol Grx4 encoded by CNAG_02950 has a role in the iron network of \textit{C. neoformans}. (N. Rouhier, et al., 2010). To test this hypothesis, I validated the Cir1-Grx4 interaction by protein-protein interaction methods both in vitro and in vivo. I also tested the function of Grx4 in iron homeostasis by analyzing the phenotypes associated with deletion of the conserved Grx domain in Grx4. Growth of the mutant was tested in different concentrations of iron and the influence of Grx4 on expression of virulence factors was tested in vitro. To facilitate the analysis of genes and cellular functions that might be influenced by GRX4, I further explored the influence of Grx4 on the transcriptome of \textit{C. neoformans} by RNA-Seq with subsequent validation of selected genes by quantitative Real-Time PCR (qRT-PCR). I continued our analysis on Grx4 by using the RNA-Seq data to characterize isoforms of the \textit{GRX4} transcript predicted from the genome annotation, and and used a genetic approach test whether larger deletions of \textit{GRX4} locus caused lethality. Overall, these studies reveal important roles for Grx4 in iron homeostasis and virulence in \textit{C. neoformans}.

2.2 Materials and methods

2.2.1 Yeast strains, plasmids and growth media

The serotype A strain H99 of \textit{C. neoformans} var. \textit{grubii} was used as the WT strain along with the \textit{cir1} mutant, \textit{grx4} mutant, and the complemented strain \textit{grx4\Delta::GRX4}. A
stable diploid *C. neoformans* strain AI187 was kindly provided by Dr. Alex Idnurm for progeny analysis. The strains were maintained on YPD medium (1% yeast extract, 2% peptone, 2% dextrose and 2% agar). Selectable markers for transformation were from plasmids pCH233 (nourseothricin resistance for *grx4Δ and cir1Δ*) and pJAF1 (neomycin resistance for the complemented strain). Nourseothricin was used for selection at 100μg ml⁻¹ and neomycin was used at 200μg ml⁻¹. Low iron medium (LIM) was prepared as described previously (S. E. Vartivarian et al., 1993). Briefly LIM was composed of 6.7g YNB plus 2% glucose in 1 L of iron-chelated H₂O using BIORAD chelex-100 and supplemented with the membrane impermeable iron chelator bathophenanthroline-disulfonic acid (BPS). The pH was adjusted to 7.2, and the medium was filter sterilized. To prepare iron-replete media, FeCl₃ was added to low-iron medium at 100 μM concentration. Cells were pre-cultured in YPD overnight at 30°C and starved in LIM for 48 hours, to make sure intracellular iron was depleted before transferring cells to LIM or iron-replete media, as described previously (W. H. Jung, et al., 2009, W. H. Jung, et al., 2008). For growth assays on solid media, ten-fold serial dilutions of cells were spotted onto plates indicated in the text with or without the supplemented iron sources. Plates were incubated at 30°C for two days before being photographed.

**Table 2.1: Strains used in Chapter 2**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Provided/Prepared by</th>
</tr>
</thead>
<tbody>
<tr>
<td>6X His::Grx4</td>
<td><em>E. coli</em> BL-21™ expressing His-tagged recombinant Grx4</td>
<td>Rodrigoun Attarian</td>
</tr>
<tr>
<td>MBP::Cir1</td>
<td><em>E. coli</em> BL-21™ expressing MBP-tagged recombinant Cir1</td>
<td>Rodrigoun Attarian</td>
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</table>
### Table 2.2: Primer sequences for strain construction

<table>
<thead>
<tr>
<th>Allele constructed</th>
<th>Primer identification</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>6X His::Grx4</td>
<td>Ndel-RA1-F</td>
<td>AAACATATGATGAACTCTACACCTACGC</td>
</tr>
<tr>
<td></td>
<td>RA1-R-HindIII</td>
<td>AAAAGCTTTTACTCCGTCTTGCCCTC</td>
</tr>
<tr>
<td>MBP::Cir1</td>
<td>Ndel-RA3-F</td>
<td>AAAAAAACATATGATGCCGAAAAGAATACCTCC</td>
</tr>
<tr>
<td></td>
<td>RA1-R-EcoRV</td>
<td>AAAGATATCCTAAGGAAGCAGGCTGAGC</td>
</tr>
<tr>
<td>Allele constructed</td>
<td>Primer identification</td>
<td>Primer sequence</td>
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<td>--------------------</td>
<td>-----------------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>grx4Δ::GRX4</td>
<td>SS-A</td>
<td>GTTTCTACATCTCTTTCCGTGTTAATACAGAGACCTGACCCGATGACGCTGAAAAACGCACG</td>
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<td>SS-B</td>
<td>CCGCGACGTGGTTGGCTCTGGCTTAATGAATGGGGCGCG TGTTGTTGATTGTGATGGGGAT</td>
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<tr>
<td></td>
<td>SS-7</td>
<td>AGGACGTCACTCTCCGAGCTTAACAACC</td>
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<td></td>
<td>SS-C</td>
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<td></td>
<td>SS-1</td>
<td>GCGAGATGGGGCACATCTTTATGTGACC</td>
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<tr>
<td></td>
<td>SS-D</td>
<td>CTGCGAGGATGAGCTG</td>
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<td></td>
<td>SS-E</td>
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<td>Cir N-F Y2H</td>
<td>ATGCCCGAAGACCAATACCTCGGC</td>
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<tr>
<td>pDEST™ 32</td>
<td>Cir N-R-Y2H</td>
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<td>Y2H-Grx4-F</td>
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<td></td>
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<td>Grx4B-6</td>
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<td></td>
<td>Grx4B-9PO</td>
<td>CGGTTGACACGAGACTTGATGTGGAG</td>
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<td></td>
<td>Grx4B-10PO</td>
<td>CCTCCCGTTCGCAACCTAATCCT</td>
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<td>Allele</td>
<td>Primer identification</td>
<td>Primer sequence</td>
</tr>
<tr>
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<td>-----------------------</td>
<td>-------------------------------------</td>
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<tr>
<td>grx4Δ</td>
<td>NAT3L-long3</td>
<td>GCCACTCGAATCCTGATGCTATTATGAGT</td>
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<tr>
<td>(Continued)</td>
<td>NAT5R-long1</td>
<td>CAGCAACGCCGTTGAATCCTCAGGATCTTTC</td>
</tr>
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2.2.2.1 Deletion of the conserved Grx domain to create a grx4 mutant and generation of grx4Δ::GRX4 complemented strain

A grx4 disruption mutant was constructed by homologous recombination using a nourseothricin acetyltransferase (NAT) marker linked to 5' and 3' flanking sequences of GRX4 C-terminal domain by three-step overlapping PCR (primer list 2.2). The overlap PCR product was biolistically transformed into the WT strain H99, and deletion was confirmed by PCR and Southern blot hybridization as previously described (R. C. Davidson et al., 2002, D. L. Toffaletti et al., 1993, J. H. Yu et al., 2004) (M. Chayakulkeeree et al., 2008). Briefly, genomic DNA for Southern blot analysis was prepared using cetyl trimethyl ammonium bromide (CTAB) phenol-chloroform extraction (Figure 2.1).

To reconstitute the deleted region of GRX4 in the mutant strain in locus, a genomic DNA fragment containing 1.1 Kb of upstream promoter region and a 1.7 Kb region carrying the deleted portion of GRX4 gene was amplified by PCR. This PCR fragment was fused with the neomycin (NEO') selectable marker (1.9 Kb) at its C terminus in an overlap PCR reaction. The overlap PCR product was introduced into the grx4 mutant by biolistic transformation. Targeted integration was confirmed by PCR and phenotypic overlay assays with TTC were performed to confirm restoration of the grx4 mutant phenotypes.
2.2.2.2 Genetic analysis of \textit{GRX4} in a haploid and a diploid \textit{C. neoformans} background

A deletion mutant lacking the complete \textit{GRX4} open reading frame was constructed by homologous recombination using a neomycin (NEO) marker linked to 5’ and 3’ flanking sequences of \textit{GRX4} by three-step overlapping PCR. The overlap PCR product was biolistically transformed into haploid H99 strain, or diploid strain AI187 (a kind gift provided by Dr. Idnurm School of BioSciences, University of Melbourne). Deletion of full-length \textit{GRX4} in the haploid H99 background was assessed by PCR, growth on YPD supplemented with 100 μg/ml neomycin, and a TTC overlay assay to evaluate cell surface reductase activity (R. Hassett and D. J. Kosman, 1995, M. Ogur et al., 1957). Sporulation of the transformed diploid strains was performed on V8 mating medium in the dark at room temperature for 5 days as described previously (R. A. Sia et al., 2000). The appearance of haploid meiotic progeny was evaluated by observing the peripheries of test spots on V8 plates and spores were collected from prominently formed hyphae followed by random spore analysis. To test for full deletion of \textit{GRX4}, basidiospores were incubated on YDP at 30°C and transferred to YPD plate containing NEO. Deletion was further analyzed by growth in presence of NEO, by the TTC assay and by PCR.
Figure 2.1: Construction of grx4 mutant and complemented grx4Δ::GRX4 strain, and confirmation of grx4 mutant by Southern Hybridization. Schematic representation of the WT loci for GRX4 open reading frame (A), nourseothricin resistance gene used to replace the 1kB of C-terminal Grx domain (B) and construct used to generate grx4Δ::GRX4 reconstituted strain (D). The blue box represents the GRX4 reading frame. NAT and NEO represent the nourseothricin and neomycin resistance cassettes, respectively. The grx4Δ strain contains deletion of C-terminal Grx domain in a haploid WT-H99 background (B). In panel (A) WT-H99 genomic DNA was digested with BglII and XbaI restriction enzymes. In panel (D), genomic DNA from grx4 mutant was digested with the same combination of restriction enzymes. The digested DNA was hybridized with a probe amplified from the
DNA sequence upstream of the *GRX4* open reading frame (A and B). Panel C illustrates genomic hybridization results with the probes indicated in panels A and B and confirmed disruption of *GRX4* and deletion of C-terminal conserved Grx domain. Neomycin resistant gene was used for complementation of *grx4* mutant reconstitution of Grx deleted domain back in locus by biolistic transformation (D).

### 2.2.3 *In silico* protein analysis

The Grx4 amino acid sequence from the *C. neoformans* serotype A strain H99 was used to search the *C. neoformans* serotype A genome sequence of the H99 strain (http://www.broadinstitute.org/annotation/genome/cryptococcus_neoformans, 2009) as well as other serotypes of *C. neoformans*, fungal species and eukaryotes. (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins). Multiple sequence alignments were performed using Clustal W (http://www.ebi.ac.uk/clustalw/).

### 2.2.4 Gene cloning, protein expression and purification

Coding sequence region of Grx4 (1-1275 bp) and N-terminal region of Cir1 (1-1224 bp) from *C. neoformans* H99 strain (http://www.broadinstitute.org/annotation/genome/cryptococcus_neoformans, 2009) were amplified from cDNA template using oligonucleotides detailed in Table 2.2.

*GRX4* cDNA was purified and subsequently cloned into pET-28b (Invitrogen) using NdeI and HindIII restriction enzymes. The Grx4/pET-28b plasmid was then transformed into *Escherichia coli* DH5α cells and once the identity of the gene was confirmed by sequencing, it was transformed into *E. coli* BL-21 (DE3) cells for expression of the His6x-tagged protein as described previously (R. Attarian et al., 2009). The N-terminal coding sequence region of Cir1 was re-amplified from a previously sequenced *CIR1* full-length
cDNA clone in pET-32a and subcloned into pMAL-c5X using Nde1 and EcoRV restriction enzymes and Cir1 produced as a maltose-binding-protein-tagged protein in *E. coli* BL-21 (DE3). Luria–Bertani medium supplemented with 100 μg of ampicillin was used for bacterial cultures. Overnight cultures were prepared by picking a single colony for the *E. coli* BL-21 (DE3) strains harboring the Grx4/pET-28b or Cir1/pMAL-c5X plasmids, and inoculating liquid medium for growth at 37°C. The next day, the cultures were diluted 1:100 and the cells were induced with 0.3 mM IPTG (isopropyl β-D-thiogalactoside) at an OD600 of 0.6–0.8. Induced cells were shaken overnight at room temperature (25°C), harvested and stored at −20°C until further processing. The N-terminal MBP-tagged Cir-1 was purified by affinity chromatography using maltose resin (New England Biolabs) and His-6x-tagged Grx4 was purified using a Ni²⁺-NTA (Ni²⁺-nitrilotriacetate) resin (Qiagen) following the manufacturer’s instructions. Proteins were separated by SDS-PAGE (12%) and visualized with Coomassie staining.

### 2.2.5 Protein–protein interaction

#### 2.2.5.1 AlphaScreen™ binding assays:

AlphaScreen™ assay (Amplified Luminescent Proximity Homogeneous Assay, PerkinElmer Life Sciences) is a bead-based technology that was designed to measure the proximity of donor and acceptor beads conjugated to analytes of interest (E. F. Ullman et al., 1994). The binding between Grx4 and Cir1 was measured using an AlphaScreen histidine (nickel chelate) detection kit (6760619M) and AlphaScreen assay buffer containing 25 mM HEPES (pH 7.5), 100 mM NaCl, and 0.1% BSA. Specifically, purified N-terminal MBP-tagged Cir1 was first biotinylated using the EZ-link Biotinylation Kit (Pierce). Then the biotinylated MBP-tagged Cir1 or biotinylated purified MBP tag (as
control) were serial diluted in assay buffer in white opaque 384-well microplates (PerkinElmer), followed by addition of streptavidin-coated donor beads. The reaction mixes were incubated for 30 minutes at room temperature. In the next step, purified 6x His-tagged recombinant Grx4 was added to the reaction mix followed by addition of nickel-chelated acceptor beads, which bind the 6x His tag fused to the analyte. An unrelated purified protein from *Mycobacterium tuberculosis*, His-tagged Rv0323c, was used as a control. The reactions were incubated for 1 hour at room temperature. The reaction kinetics was then monitored on the Fusion-α-HT Multimode Microplate Reader (PerkinElmer) for luminescence signal generated from protein-protein interaction (counts per seconds). When the donor and acceptor beads were brought into proximity by the interaction between N-terminal MBP- Cir1 and 6x His-tagged Grx4, and the sample was excited with a laser at 680 nm to release singlet oxygen molecules. Within its 4-millisecond half-life, singlet oxygen can diffuse approximately 200 nm in solution. This enables the excitation of the Alpha acceptor beads, which then elicit a strong emission of light at a shorter wavelength (520-620 nm) to be counted and registered by FusionAlpha plate reader (PerkinElmer), as counts per second. To determine the approximate dissociation constant values, the data were fitted to a hyperbolic function using Graphpad®.

2.2.5.2 Yeast two-hybrid assay

The assays were performed using the ProQuest Two hybrid system with Gateway Technology Manual, Invitrogen Life Technologies Inc. according to manufacturer’s protocols as previously described (G. Hu et al., 2015). Briefly, *GRX4* (1-1648 bp) and the N-terminal region of *CIR1* (1-1203 bp) were PCR amplified from *C. neoformans* cDNA using the primers listed in Table 2.2, cloned into pDEST-22 (*GAL4* activting domain) and pDEST-
32 (GAL4 DNA binding domain) vectors, respectively and co-transformed into MaV203 yeast competent cells.

The growth of MaV203 yeast expressing both bait and prey vectors was tested on synthetic complete medium (0.7% Yeast nitrogen base without amino acids (Difco), 2.0% glucose, 0.07% synthetic complete selection medium mix (Sigma), 1.7% bacto agar (Difco), pH 5.6) lacking leucine and tryptophan to select for each vector, and histidine and uracil to test for an interaction. Empty pDEST-32 and pDEST-22 vectors were used as negative controls. The physical interaction between the encoded proteins in these plasmids was tested by assessing restoration of uracil and histidine prototrophy.

2.2.6 Iron-related phenotypic analysis

An overlay assay to examine the reduction of TTC was performed to evaluate cell surface reductase activity as described previously (R. Hassett and D. J. Kosman, 1995, M. Ogur, et al., 1957). Briefly, cells grown in YPD were harvested and diluted with sterile ddH2O. $1.0 \times 10^6$ cells were spotted on agar medium and incubated at 30°C for 1 day. The colonies were then overlaid with agarose (1.5% in PBS) containing 0.1% TTC. The development of red color on the colony spots was monitored and photographed. Phleomycin sensitivity was tested by spotting $1.0 \times 10^6$ cells onto YPD medium containing 0.25 μg/ml phleomycin with subsequent incubation at 30°C for 1 to 2 d. To determine sensitivity to iron-restricted or to iron-overload conditions, $10^6$ and 10-fold serial dilution cells were spotted onto YPD medium (containing 400 mM BPS) with or without 250 μM FeCl$_3$ and were grown at 30°C for 2 days.
2.2.7 Virulence factor assays

Capsule formation was examined by DIC. For this assay, cells were pre-grown in YPD, washed twice with low iron water (Li-H₂O), diluted to 10⁶ cells/ml in Capsule Inducing media (CIM), and grown for 24 hours at 30°C. CIM was prepared by dissolving 5g glucose, 5g asparagine, 4.78g HEPES, 0.4g K₂HPO₄, 0.25g CaCl₂.2H₂O, 0.08g MgSO₄.7H₂O, 1.85g NaHCO₃, add 1ml of 1000X salt stock solution in 1L of Li-H₂O, pH adjusted to 7.2 using 1 M MOPS made with low iron water, sterilized and 100ul sterile thiamine (4 mg/ml) added. The cells were then resuspended in 1/10th volume of CIM, mixed 1:1 in India ink and visualized by negative staining on a Zeiss Axioplan 2 Imaging microscope by DIC. Melanin production was examined on l-3,4-dihydroxyphenylalanine (l-DOPA) agar containing 0.1% glucose. Cells were pre grown in YPD, washed once, counted, and adjusted to 2 × 10⁶ cells ml⁻¹ in YPD. Serial dilutions were performed, and 5 μl of each dilution was spotted onto agar medium. Plates were incubated at 30°C. The ability of the grx4 mutant to grow at host temperature was tested by plating on solid media at 37°C.

2.2.8 Analysis of growth in liquid media

For growth assays in liquid media, a final concentration of 5 × 10⁴ cells ml⁻¹ was inoculated in the medium with or without supplemented iron sources. Briefly, strains (WT, grx4 mutant, grx4Δ::GRX4 and cir1 mutant) were pre-grown in triplicate cultures of 5ml of YPD overnight at 30°C, the cells were washed twice with low iron water, diluted 1:100, and incubated in low iron YNB at 30°C for an additional 48 h to eliminate any iron carryover from the rich medium. The cells were then harvested and washed twice with low iron water. The cells were counted and 10⁴ cells were transferred to LIM alone or LIM supplemented with 100 μM FeCl₃ (Sigma). Growth of the strains was also assessed by a
high-throughput 96-well microplate assay using the Infinite M200 PRO plate reader (Tecan, Austria). For this assay, iron-starved cells were inoculated in 200 µl of YNB-LIM with or without supplemented iron sources for 3 to 4 days at 30°C (final inoculum of 10^4 cells). In addition, the same experiment was performed by measurement of the optical density of high volume cultures (50 ml) at 600 nm using a DU530 Life Science UV/Vis spectrophotometer (Beckman). In a separate experimental set up to test the influence exogenous GSH on growth phenotype of strains in presence of iron, iron-starved strains (WT, grx4 mutant, grx4Δ::GRX4 and cir1 mutant) were pre-grown in the presence of iron to an O.D.₆₀₀ of 0.3. These pre-treated cells were diluted, and 10^4 cells were re-introduced to fresh LIM supplemented with 100 µM FeCl₃ in with/without 5 mM final concentration of glutathione.

2.2.9 Virulence assay

For virulence assays, female BALB/c mice (4–6 weeks old) were obtained from Charles River Laboratories (Ontario, Canada). The WT, grx4 mutant and grx4Δ::GRX cells were grown in YPD overnight at 30°C, washed in PBS and re-suspended at 1.0 × 10^6 cells ml⁻¹ in PBS. Inoculation was by intranasal instillation with 50 µl of cell suspension (inoculum of 2.0 × 10^5 cells per mouse). Groups of 10 mice were inoculated for each strain. The status of the mice was monitored twice, daily post inoculation. For the determination of fungal burdens in organs, infected mice were euthanized by CO₂ inhalation and organs were excised, weighed and homogenized in 1 ml of PBS using a MixerMill (Retsch). Serial dilutions of the homogenates were plated on YPD agar plates containing 35 µg ml⁻¹ chloramphenicol and CFUs were counted after incubation for 48 h at 30°C.
Furthermore, fungal load distribution in different tissues of the infected mice was determined. Mice reaching the endpoint were euthanized by CO₂ asphyxiation, and fungal loads in different tissues of the mice including the lungs, kidney, liver, spleen, brain and blood were determined. Tissues were aseptically removed and immersed in PBS. Organs were homogenized using an automated tissue homogenizer. The samples were serially diluted in PBS and plated on YPD supplemented with 35 μg/mL chloramphenicol. After two days of incubation at 30°C, the colony forming units (CFUs) were counted manually.

2.2.10 Ethics statement

All experiments with mice were conducted in full compliance with the guidelines of the Canadian Council on Animal Care and approved by the University of British Columbia’s Committee on Animal Care (protocol A13-0093).

2.2.11 Statistical analysis

Statistical significance of mouse survival times was assessed using a single-factor ANOVA variance test. Significant differences in fungal loads were determined by a two-tailed unpaired Student t test, using GraphPad Prism 6.0 for MAC OS X (Graph-Pad Software, San Diego, CA, USA). The virulence data were analyzed with the log rank test for statistical differences.

2.2.12 RNA-Seq library preparation and bioinformatics

Comparative transcriptional profiling of the WT, grx4 mutant and cir1 mutant strains in both iron-deplete and iron-replete condition was performed by RNA sequencing. Each strain was grown in 25 ml of YPD overnight at 30°C. Cells were washed twice with low iron medium (LIM) followed by growth in LIM at 30°C for an additional 48h to eliminate any iron carryover from the rich medium. Cells were then harvested and washed twice
with LIM. The cells were counted and transferred to 50 ml LIM or LIM+100 μM of FeCl₃ (final density of 2x10⁵ cells/ml). The cells were then grown at 30°C for another 6 h and harvested for RNA extractions. RNA was purified with the RNeasy kit (Qiagen) and treated with DNase (Qiagen) following the manufacturer’s recommendations. The quality of RNA was analyzed with an Agilent 2100 Bioanalyzer and stored at -80°C. A total of 10 µg RNA was used for the TruSeq RNA Sample Prep Kit v2 (Illumina, Inc., CA). In order to construct libraries, the libraries were sequenced on a HiSeq 2000 (Illumina, Inc., CA) using 100 bp in paired-end mode. Raw read data was quality-checked and trimmed using FastQC(http://www.bioinformatics.babraham.ac.uk/projects/fastqc/).

Trimmed reads were aligned to the C. neoformans serotype A strain H99 reference genome obtained from the Broad Institute [Cryptococcus neoformans Sequencing Project, Broad Institute of Harvard and MIT (http://www.broadinstitute.org). For aligning reads, TopHat 2.0.9 (C. Trapnell et al., 2009) was used with default settings except for adjusting the expected minimum and maximum intron lengths to 10 bp and 5000 bp, respectively. Aligned reads were assembled into transcripts and compared to the reference genome annotation with Cufflinks 2.1.1 (C. Trapnell et al., 2010). Cufflinks was further used for de novo assembly of predicted and unannotated transcript variants of GRX4. The R packages cummeRbund version 0.1.3 (C. Trapnell, et al., 2010), ggplot2 version 0.9.3 and custom scripts were used to analyze and visualize the transcript data. The merged data was collected into a consensus GTF file using Cuffmerge (version 2.02).

For Gene Ontology analyses, transcript sequences with an absolute fold change of 3 or more with a p-value of less than p<0.05 between WT and grx4 mutant under low iron and iron replete conditions were analysed using Gene Ontology (GO) terms available on
locally installed Blast2GO (Conesa et al., 2005). The *C. neoformans* H99 reference genome was used for gene annotation [Cryptococcus neoformans Sequencing Project, Broad Institute of Harvard and MIT (http://www.broadinstitute.org May 2014)]. GO term enrichment datasets were visualized using the R package ggplot2.

### 2.2.13 Quantitative Real-Time PCR

To examine gene expression, three biological replicates of the WT and *grx4* mutant and *cir1* mutant strains were grown in 5 ml of YPD medium overnight at 30°C. Cells were washed twice in chelex-treated water followed by growth in YNB-LIM for two days to starve the cells for iron. Cells were then washed and grown either in 5 ml YNB-LIM (with or without 100 μM FeCl₃) for six hours at 30°C. Samples were collected in triplicate and flash frozen in liquid N₂, and stored at -80°C.

Total RNA was extracted using the QIAGEN RNeasy Mini Kit and subsequently treated with RNase-free DNase. cDNA was synthesized using oligo(dT) primer and Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit. The resulting cDNA was used as template for quantitative real-time PCR using iQ SYBR Green Supermix (Bio-Rad) according to the manufacturer’s specifications. The iCycler iQ multicolor real-time detection system was used as the fluorescence detector.

The relative gene expression was quantified based on the 2^ΔΔC_Δ_ method with 18S rRNA as an internal control for data normalization. Primers (Table 2.3) were designed using Primer3 v.4.0 (http://bioinfo.ut.ee/primer3-0.4.0) and targeted to the 3′regions of transcripts. Relative gene expression was quantified using the Applied Biosystems™ 7500 Fast Real-time PCR system and fold changes were determined based on comparison of gene expression to the WT-H99 strain.
Table 2.3: Primer sequences for quantitative qRT-PCR

<table>
<thead>
<tr>
<th>Primer identification</th>
<th>Primer sequence</th>
<th>Target Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>sit1-f1</td>
<td>GCACGGAGGAGGTCGTTGTA</td>
<td>SIT1 (CNAG_00815)</td>
</tr>
<tr>
<td>sit1-r1</td>
<td>GCTCAGCGAGGTGTGCAAA</td>
<td></td>
</tr>
<tr>
<td>str3-f1</td>
<td>TCGCCGCTTCTCTCCAA</td>
<td>STR3 (CNAG_07387)</td>
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<tr>
<td>str3-r1</td>
<td>GCTTGCCTGGACGGTAAGA</td>
<td></td>
</tr>
<tr>
<td>mirB-f1</td>
<td>GCTCAGCGAGGTGTGCAAA</td>
<td>MIRB (CNAG_07751)</td>
</tr>
<tr>
<td>mirB-r1</td>
<td>CGACATTCTCGACAAATGCAAA</td>
<td></td>
</tr>
<tr>
<td>cfo1-f1</td>
<td>GGACCTTGAGCGCTCAAA</td>
<td>CFO1 (CNAG_02958)</td>
</tr>
<tr>
<td>cfo1-r1</td>
<td>CAAGCGCGCCAATCG</td>
<td></td>
</tr>
<tr>
<td>cft1-f1</td>
<td>TCGACCCGGCTCTACCA</td>
<td>CFT1 (CNAG_02959)</td>
</tr>
<tr>
<td>cft1-r1</td>
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<td>catalase</td>
</tr>
<tr>
<td>cat3-r1</td>
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<td>(CNAG_05015)</td>
</tr>
<tr>
<td>cat4-f1</td>
<td>CGCAATGGTTGGTTGAGACA</td>
<td>catalase</td>
</tr>
<tr>
<td>cat4-R1</td>
<td>GCCATTCTCTGGACCACACT</td>
<td>(CNAG_00575)</td>
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<td>18S-RT-F</td>
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</tr>
<tr>
<td>18S-RT-R</td>
<td>ACTCGCTTGCTCAGTCAGTG</td>
<td>(CNAG_03246)</td>
</tr>
</tbody>
</table>

2.3 Results

2.3.1 The monothiol glutaredoxin Grx4 binds Cir1

2.3.1.1 Grx4 was identified as a potential Cir1-associated protein

The monothiol glutaredoxin Grx4 encoded by CNAG_02950 was originally identified by mass spectrometry (MALDI-TOF, UBC) in a preliminary experiment performed by Dr. Horacio Bach to identify Cir1-associated proteins. The proteins identified by Dr. Horacio Bach in this experiment are listed in Appendix A: Table A1, and Figure A1.). The identified
peptides that matched the CNAG_02950 polypeptide predicted from the *C. neoformans*
serotype A genome sequence

(http://www.broadinstitute.org/annotation/genome/cryptococcus_neoformans).

Database version 2009, are highlighted in Figure 2.2. The putative interaction of Grx4 with Cir1 observed in this preliminary experiment was intriguing given the known interactions of monothiol glutaredoxins with iron regulatory factors in *S. cerevisiae* and *S. pombe* (Chapter 1).

**Figure 2.2: Identified peptides obtained from MS analysis.** Peptides that matched the CNAG_02950 polypeptide are highlighted in yellow.

**2.3.1.2 Production of recombinant Grx4 and Cir1 in *E. coli* for interaction studies**

As an initial step to validate the interaction between Grx4 and Cir1, each of the proteins were expressed in *E. coli* and purified by affinity chromatography. As a first step, cDNA from the WT strain H99 was produced as described in the Materials and Methods (section 2.2.13.1). The Grx4 full-length cDNA was then obtained by PCR and cloned into the expression vector pET-28b. Following overexpression in *E. coli* BL-21, the protein was
purified as a recombinant 6x His-tagged polypeptide by Ni-NTA affinity chromatography (Figure 2.3.). (See Material and Methods, section 2.2.4).

Figure 2.3: Purification of the His$_6$-tagged Grx4 polypeptide. The 6x His-tagged recombinant Grx4 polypeptide was resolved by 12% SDS-PAGE and detected with α-His antibody. The Precision Plus Protein™ Dual Color Standards (#161-0374EDU) are indicated on the right side (B).

The Cir polypeptide is 952 amino acids long and contains a cysteine-rich domain (amino acids 162-189) and a conserved zinc-finger domain (amino acids 307-359) in the
N-terminal region (amino acids 1-408). It has been shown previously that the zinc-finger domain in the N-terminal region of Cir1 is critical for function and required for the stability of Cir1. Of note, the contribution of the C-terminal region to the function of Cir1 is not known (W. H. Jung and J. W. Kronstad, 2011). In this study, several attempts to produce the full-length version of Cir1 were not successful; however, I was able to successfully produce the N-terminal version of Cir1 tagged with the maltose binding protein (MBP) (See Materials and Methods, section 2.2.4). As shown in Figure 2.4, it was necessary to concentrate the fusion protein with an Amicon filter to separate it from contaminating proteins or degradation products of lower mass. Together, the expression approaches for Grx4 and Cir1 provided the proteins necessary for subsequent validation of interaction.
Figure 2.4: Purification of Cir1 tagged with the maltose binding protein (MBP). Lane 1 contains the eluate of the MBP-tagged recombinant N-terminal region of Cir1 following maltose affinity purification. Lane 2 contains the eluate of the MBP-Cir1 fusion protein concentrated on Amicon Ultra 50 K (Milipore) before SDS-PAGE. The lane labeled M contains the Protein Ladder Precision Plus Protein™ Dual Color Standards (#1610374EDU).

2.3.1.3 Grx4 binds Cir1 in an ALPHAScreen® protein-protein interaction assay

As mentioned in section 2.3.1.1, Grx4 was initially pulled down by Cir1 in a binding assay and identified by mass spectrometry. I sought to further validate this interaction by using the AlphaScreen® technique, an in vitro method for detecting and characterizing
protein-protein interactions. ALPHAScreen® employs photoactive donor and acceptor beads that recognize specific tags on candidate interacting proteins. In my assay, the biotinylated MBP-tagged N-terminal region of Cir1 (amino acids 1-408) and recombinant 6X His-tagged Grx4 (amino acids 1-425) were used to examine the potential interaction. An overview of this assay is illustrated in Figure 2.5.

Figure 2.5: Overview of the Grx4-Cir1 Alphascreen assay. The 6x His-tagged Grx4 protein (black) was mixed with biotinylated Cir1 (purple) at a range of concentrations. Nickel chelate acceptor (yellow) and streptavidin donor AlphaScreen beads (blue) were then added. Upon irradiation with 680 nm laser light, the donor beads produce singlet oxygen ($^{1}ΔO_{2}$) that diffuses ~200 nm. Acceptor beads that are potentially in close proximity because of interactions of the Grx4 and Cir1 proteins on their surface may react with the $^{1}ΔO_{2}$ and emit light between 520-620 nm. Emission is measured using a Fusion® αHT apparatus (PerkinElmer).
The AlphaScreen analysis demonstrated direct binding of Cir1 and Grx4 in reciprocal reactions *in vitro* (Figure 2.6). A hyperbolic curve plotting emission at 520-620 nm as a function of Cir1 concentration generated a curve fitting the 1:1 Langmuir binding model, predicted a Cir1-Grx4 dissociation constant (Kd) of 2x10^{-6}M. The purified MBP did not bind His6-tagged Grx4 in the control experiment (Figure 2.6 A). The reciprocal experiment with increasing amounts of Grx4 yielded similar results. A hyperbolic curve fitting the 1:1 Langmuir binding model and a dissociation constant (Kd) of 2.x 10^{-6} M confirmed binding between Cir1 and Grx4. I did not detect an interaction between MBP-tagged Cir1 and a recombinant protein from *Mycobacterium tuberculosis* (His6-tagged Rv0323c overexpressed in *E. coli*) in a control experiment (Figure 2.6 B). Overall, these results validated the initial interaction detected by mass spectrometry and indicated that Grx4 binds Cir1.
Figure 2.6: Binding between Cir1 and Grx4 detected by ALPHAScreen analysis. (A) Recombinant Cir1 was subjected to the ALPHAScreen assay with increasing concentrations of Grx4. The MBP served as a negative control. (B) A reciprocal experiment was performed with increasing concentrations of Cir1. His6-tagged Rv0323c protein was used as a negative control.
2.3.1.4 Grx4 binds Cir in a yeast two-hybrid assay

I further sought to confirm the interaction between Grx4 and Cir1 \textit{in vivo}. The yeast two-hybrid system was employed for this approach. The cDNAs for Grx4 and the N-terminal region of Cir1 were fused to the GAL4 activating domain (AD) in pDEST-22 (prey plasmid) and the GAL4 DNA binding domain (BD) in pDEST-32 (bait plasmid), respectively. These vectors were co-transformed into competent yeast cells (strain MaV203). Yeast cells that harbored both AD-Grx4 and BD-Cir1 were selected based on growth in the absence of leucine and tryptophan. The ability of these transformants to grow without uracil or histidine was then tested and growth used as an indication of a positive interaction between Grx4 and Cir1 (Figure 2.7).

Overall, the yeast two-hybrid assay confirmed the findings from mass spectrometry and the AlphaScreen assay in further support of the conclusion that Grx4 interacts with Cir1. As mentioned, this interaction is consistent with observed interactions between monothiol glutaredoxins and iron regulators in other fungi, including Grx4 with Fep1 in \textit{S. pombe} and Grx3/4 with Aft1 in \textit{S. cerevisiae} (M. Jbel, et al., 2011, L. Ojeda, et al., 2006). The interaction of Grx4 with Cir1, the main iron regulatory factor in \textit{C. neoformans}, raised the possibility that loss of Grx4 might lead to shared phenotypes with mutants lacking Cir1. Therefore, I continued to further investigate the association between Grx4 and Cir1 by phenotypic analysis of \textit{grx4} and \textit{cir1} mutants, as described below.
Figure 2.7: Grx4 physically interacts with Cir1 in a yeast two-hybrid assay. The constructs with BD and AD indicate the GAL4 DNA binding and activation domains fused to Grx4 and Cir1, respectively. The vector designation indicates the empty vector control.

(A) All combinations of transformants grew in the absence of leucine (Leu) and tryptophan (Trp), confirming the presence of the plasmids in the strains. (B) Yeast cells co-transformed with plasmids containing AD-Grx4 and BD-Cir1 grew in the absence of uracil (Ura) or histidine (His), confirming an interaction that allowed expression of URA3 and HIS3. As part of the detection of HIS3 expression, 3-amino-1,2,4-trazole was used at different concentrations. A strain harboring plasmids expressing the known interacting proteins Rim20 and Snf7 was used as a positive control (G. Hu, et al., 2015).
2.3.2 A defect in Grx4 impacts iron homeostasis and virulence

2.3.2.1 Identification of a conserved C-terminal glutaredoxin domain in GRX4 in *C. neoformans*

An analysis of the amino acid sequence similarity of Grx4 with other members of the monothiol glutaredoxin class of proteins revealed that Grx4 has a conserved C-terminal glutaredoxin (Grx) domain with a signature “CGFS” motif in the predicted active site (Figure 2.8 A). A Clustal Omega alignment of the amino acid sequence of the C-terminal Grx domain of the *C. neoformans* protein with four selected Grx sequences from other organisms is shown in Figure 2.8 B. According to a Blastp analysis ([https://blast.ncbi.nlm.nih.gov/Blast.cgi](https://blast.ncbi.nlm.nih.gov/Blast.cgi)), the C-terminal domain in Grx4 in *C. neoformans* shares 44% amino acid sequence identity with C-terminal region in Grx4 from *S. pombe*, 41% with Grx4 from *S. cerevisiae*, 60% with Grx3 from *S. pombe*, and 51% identity with Grx3 from *Homo sapiens* (Figure 2.8 C). Of note, the monothiol Grx domain with the CGFS active site motif is highly conserved and is known to be required for Fe-S cluster binding and trafficking, and the regulation of iron homeostasis in fungi ([J. Encinar del Dedo, et al., 2015, H. Li and C. E. Outten, 2012](#)). Combined with its interaction with the iron regulator Cir1, the sequence analysis supports a possible role for Grx4 in iron-related processes in *C. neoformans*. 
Figure 2.8: Domain structure of *C. neoformans* Grx4. (A) The N-terminal Trx domain consisting of amino acids 193-313 and the Grx domain consisting of amino acids 332-399 are separated by a linker region. The active site motif of the Trx-like domain (WAxxC) and Grx-like domain (CGFS) are indicated at the top of the figure. (B) Sequence alignment of the C-terminal Grx domain of monothiol Grxs in model yeasts and human. The conserved active site motif (CGFS) is highlighted in magenta and the (*) and (: symbols indicate
identical and similar amino acids, respectively. Sequences were aligned using the CLUSTAL Omega (1.2.1) multiple sequence alignment tool (http://www.ebi.ac.uk/Tools/msa/clustalo/). Abbreviations are as follows: Cn= (C. neoformans var. grubii strain H99), Hs= (H. sapiens), Sc= (S. cerevisiae), Scp= (Schizosaccharomyces pombe). (C) A high percentage of identity in amino acid sequences in the C-terminal Grx domain across the selected species as determined by Blastp analysis is shown.

2.3.2.2 A grx4 deletion mutant has iron-related phenotypes

Next I sought to gain insights into the function of Grx4 in C. neoformans by phenotypic characterization of a grx4 mutant in which the C-terminal conserved Grx domain was deleted and replaced by a NAT resistance marker. This grx4 mutant was kindly provided by our collaborator Dr. J Lodge (Washington University, St. Louis, MO, USA). I confirmed the deletion by Southern hybridization, and also complemented the mutation by inserting the WT GRX4 gene at its original locus to generate a grx4D::GRX4 strain (see Material and Methods, section 2.2.2.1).

The grx4 mutant was tested for iron-related phenotypes including growth in the presence of elevated iron levels, cell surface reductase activity and susceptibility to the iron-dependent inhibitor phleomycin. Each assay was performed at least 3 times and representative results are presented in Fig. 2.9. A cir1 mutant was included throughout the experiments for comparison. Initially, the growth phenotype of the grx4 mutant in rich YPD medium was tested and it was found that the mutant exhibits equivalent growth to the WT strain in YPD in both plate assays and liquid culture. Therefore, YPD was used in
subsequent plate assays as the standard medium. Also, it has been shown that loss of Cir1 causes sensitivity to elevated iron levels ([W. H. Jung, et al., 2006]). We therefore tested the growth phenotype of grx4 mutant in presence of elevated iron levels in both absence or presence of the iron chelator BPS. Ten fold serial dilutions of the WT strain (H99), the grx4 mutant, the grx4Δ::GRX4 complemented strain and the cir1 mutant were grown in YPD at 30°C and spotted onto YPD+ 250 μM FeCl₃. Similar to the cir1 mutant, the grx4 mutant displayed slower growth in the presence of iron (Figure 2.9). We further tested this phenotype in the grx4 mutant by first chelating available iron in YPD by addition of 400 mM of the iron chelator BPS. All four strains, including the cir1 and grx4 mutants, the WT strain and the complemented strain, have an impaired growth phenotype in the iron-restricted condition in the presence of BPS. However, the growth of all of the strains, including the grx4 and cir1 mutants, was improved upon addition of iron thus suggesting that iron uptake is not impaired in both mutants.

It has been shown that cir1 mutants have increased cell surface reductase activity, which appears as a red colony color on media with TTC (W. H. Jung, et al., 2006). This phenotype is consistent with the derepression of transcript levels for ferric reductase genes in the cir1 mutant (W. H. Jung et al., 2010). As expected, colonies of the grx4 mutant showed an enhanced red color compared with the WT strain indicating higher cell surface reductase activity. I speculate that this phenotype is due to derepression of reductase gene transcription in the grx4 mutant similar to the cir1 mutant. Complementation of the grx4 deletion with GRX4 resulted in WT phenotypes in these assays indicating that the observed phenotypes are due to deletion of GRX4.

Excess iron is damaging due to formation of reactive oxygen species via the Haber-
Weiss/Fenton reaction (J. A. Imlay, 2008, J. A. Imlay, et al., 1988). In this context, phleomycin is a glycopeptide antibiotic, which causes DNA damage in the presence of ferrous iron and oxygen due to the production of reactive oxygen species (L. F. Povirk et al., 1981). It was also shown previously that a *cir1* mutant displays susceptibility to phleomycin (W. H. Jung, et al., 2006). Similar to *cir1* mutant, we found that the *grx4* mutant exhibits enhanced sensitivity to phleomycin relative to the WT and complemented strains. Overall, the accumulated iron-related phenotypes observed in the *grx4* mutant indicated that Grx4 is required for growth in the presence of iron and overall maintenance of iron homeostasis in *C. neoformans*.

Figure 2.9: A *grx4* mutant displays iron-related phenotypes similar to the *cir1* mutant. Spot assays starting with a culture of 2x10^8 cells per ml (10^6 cells in 5μl spotted) and 10-fold dilutions from left to right were performed on YPD media containing the various conditions indicated below the panels. These conditions include: (A) YPD without additions to demonstrate similar growth of the *grx4* and *cir1* mutants; (B) elevated iron levels (YPD+ 250 μM FeCl₃) in which the *grx4* and *cir1* mutants show sensitivity; (C) an iron restricted condition (-Fe, 400 μM BPS) in which both mutants have impaired growth; (D) an elevated iron level (+Fe, 400 μM BPS + 250 μM FeCl₃) in *grx4* and *cir1* mutants.
display sensitivity; (E) cell surface reductase activity as indicated by the red colony color in the presence of TTC, in which the grx4 and cir1 mutants display increased activity; and (F) susceptibility to phleomycin (0.25 g/ml) for the grx4 and cir1 mutants.

I further analyzed the growth phenotypes of the grx4 and cir1 mutants, along with the WT strain and the grx4Δ::GRX4 complemented strain in the iron-restricted condition in liquid assays. The strains were first grown for two days in low-iron medium (LIM-YNB containing 150 μM of the iron chelator BPS) to exhaust intracellular iron stores. Supplementation of this medium with 25 μM, 50 μM or 100 μM ferric iron generated iron-replete conditions. After iron starvation, I tested the growth of all of the strains in YNB-LIM without or with the various levels of FeCl₃. The growth assays revealed that the grx4 mutant does not respond to the presence of iron at lower concentrations (25 μM, 50 μM) (Figure 2.10 A). Analysis of the growth of the grx4 mutant in the presence of 100 μM FeCl₃ showed that this concentration of iron in liquid culture supports growth. However, I did observe that the grx4 mutant had a prominent growth defect compared with the parental WT strain, the cir1 mutant and the grx4Δ::GRX4 complemented strain (Figure 2.10). As expected, none of the strains grew in LIM after 48 hours of pre-starvation for iron (Figure 2.10). Overall, these results indicated that Grx4 is required for robust growth in the presence of iron and further highlighted the contribution of Grx4 to iron homeostasis in C. neoformans. Based on the iron-related phenotypes, I speculated that grx4 mutant might be particularly susceptible to oxidative stress upon iron starvation and/or iron repletion leading to impaired growth. It has been shown that GSH protects against detrimental effects of iron-deficiency and iron toxicity due to its antioxidant properties (L. M. Milchak
and J. Douglas Bricker, 2002) (L. Ramirez et al., 2013). This prompted me to test the effect of GSH on growth defect of grx4 mutant in presence of iron after a period of iron starvation. Interestingly, the growth defect of the grx4 mutant was rescued by exogenous GSH as shown in Figure 2.11.

This observation prompted us to investigate the potential physiological role of glutathione in iron homeostasis in C. neoformans, which has not been investigated in pathogenic fungi.
Figure 2.10: Grx4 is required for growth in iron-depleted media supplemented with FeCl₃. The strains were pre-starved for iron in LIM and grown in LIM supplemented with increasing concentrations of FeCl₃: 25 µM (A), 50 µM (B), 100 µM (C). Growth was monitored by measuring the optical density at 600 nm. Data are presented as the average± S.D. As expected, none of the pre-starved strains grew in the absence of iron (LIM).
Figure 2.11: Addition of GSH restored growth of grx4 mutant under iron-replete conditions. The strains were pre-starved for iron in LIM and pre-grown in LIM supplemented with 100 μM FeCl₃ for 12 hours. Cells were washed and re-introduced to LIM+ 100 μM FeCl₃ (A) or LIM+ 100 μM FeCl₃ + 5mM GSH dissolved in in 25 mM Hepes buffer pH 7.4 (B). Growth was then monitored by measuring the optical density at 600 nm for 72 hours. The experiments were performed three times and the data are presented as the average± S.D.

2.3.2.3 Grx4 is required for inhibition of iron uptake in the presence of iron

The iron-related phenotypes of the grx4 mutant prompted me to investigate which C. neoformans genes involved in iron uptake might be influenced by loss of GRX4. I first compiled a list of genes that are known to influence iron uptake in C. neoformans including ferric reductases (FRE1-6), siderophore transporters (SIT1, STR3, MIRB), components of the high-affinity uptake system (CFT1, CFO1), and candidate genes for a low-affinity uptake system (CFT2, CFO2). I next compared the transcript levels of these genes in the WT strain
and the grx4 mutant under iron-deplete or iron-replete conditions by quantitative Real-Time PCR (q-RT-PCR) (See Materials and Methods, section 2.2.12). Under low-iron conditions, I did not detect significant differential transcript levels for the genes of interest in the grx4 mutant compared to the WT strain. However, under the high-iron condition, I found that the transcripts for the genes encoding the ferric reductases FRE2 and FRE6, the siderophore transporters, and the high-affinity uptake system were highly upregulated in the grx4 mutant compared to WT (Figure 2.11). Together, these results indicated that Grx4 is required for inhibition of the expression of iron acquisition functions in C. neoformans in high iron conditions. Also, loss of Grx4 leads to constitutive de-repression of genes that are normally upregulated in the WT strain under low-iron conditions to provide a sufficient amount of iron for Fe-dependent pathways. This is in agreement with the iron-related phenotypes observed in the grx4 mutant, including sensitivity to elevated iron levels. It is likely that de-repression of iron uptake/acquisition under high iron levels in the grx4 mutant leads to iron toxicity and increased oxidative stress; this in turn might account for the impaired growth of the grx4 mutant in the presence of iron.
**Figure 2.12:** Grx4 has a negative regulatory influence on the transcript levels for iron acquisition functions. Quantitative RT-PCR was used to measure the levels of transcripts of candidate genes in the *grx* mutant compared to the WT strain in iron-replete conditions. The data were normalized using 18S rRNA as an internal control and are presented as relative expression in comparison to the WT value set at 1. The data from 3 separate experiments (biological replicates), each of which had 3 technical replicates was combined, and the bars represent standard deviations.
2.3.2.4 Grx4 influences the virulence of *C. neoformans*.

2.3.2.4.1 The *grx4* mutant displays virulence-related phenotypes

The polysaccharide capsule is one of the major virulence traits of *C. neoformans* and, given that iron depletion results in an enlarged capsule, I evaluated the effect of *GRX4* disruption on capsule formation. It had been shown previously that a *cir1* mutant is acapsular in both serotype D and serotype A strains (W. H. Jung, 2006). Similar to the *cir1* mutant, I did not detect a capsule on cells of the *grx4* mutant; in contrast, both the WT and complemented strains displayed large capsules (Figure 2. 12 A). These results demonstrated that Grx4 is required for capsule formation, most likely through its influence on iron homeostasis.

I next examined whether other important virulence traits such as the ability to grow at host temperature (37°C) and melanin formation were altered in the *grx4* mutant. The growth of the *grx4* and *cir1* mutants resembled the WT and reconstituted *grx4Δ::GRX4* strains at 30°C, but both mutants grew poorly at 37°C (Figure 2. 13). It is known that Cir1 links iron regulation and expression of *LAC1* *C. neoformans* (W. H. Jung, 2006). Here, I tested formation of melanin on DOPA medium at 0.1% glucose in the *grx4* mutant and found that compared to WT, the *grx4* mutant produced a slightly reduced amount of melanin represented by the light grey color pigment in *grx4* mutant compared to the black one in WT (Figure 2.13 C). Overall these results show that Grx4 influences major virulence-associated traits in *C. neoformans*.
Figure 2.13: A grx4 mutant does not express virulence-related traits. Loss of the C-terminal Grx domain in a grx4 mutant leads to virulence-related phenotypes including loss of capsule formation (A), poor growth at 37°C (B) and altered melanin production (C).
2.3.2.4.2 Deletion of *grx4* abrogates virulence in a mouse model of cryptococcosis

My phenotypic analysis including the impact of the *grx4* deletion on virulence-related phenotypes strongly suggested that the mutant would have a defect in its ability to cause disease in mice. This prediction was tested by inoculating 10 female BALB/c mice intranasally with cells of the WT strain, the *grx4* mutant or the complemented strain. All mice infected with the WT and complemented cells succumbed to infection between days 18 and 21, while the mice infected with the *grx4* mutant did not show disease symptoms and survived for the duration of the experiment (i.e., 60 days) (Figure 2.14). These results indicated that *Grx4* is critical for the virulence of *C. neoformans* and I speculate that due to the significance of Grx4 in iron homeostasis and role of iron in regulation in virulence, Grx4 links iron homeostasis and virulence.

![Figure 2.14: loss of GRX4 abolished virulence.](image)

**Figure 2.14: loss of GRX4 abolished virulence.** Ten female BALB/c mice were infected intranasally with the WT strain (black triangles), the *grx4* mutant (pink circles) or the complemented strain (filled brown triangles). The survival of the mice is shown versus
time in days. Statistical analysis was performed using ordinary one-way ANOVA. The (***) symbol represents significant differences with a $P < 0.0006$. This experiment was performed in collaboration with Dr. Guanggan Hu and Dr. Melissa Caza.

Furthermore, the mice from each group were used at the time of sacrifice to analyze the distribution of fungal cells in different tissues including the lungs, kidney, liver, spleen, brain and blood. The numbers of fungal cells in the tissues of mice infected with WT or the complemented strain were comparable, but the grx4 mutant cells were 2-4 orders of magnitude lower than the WT level suggesting a defect in dissemination and/or colonization of organs by the mutant. (Figure 2.15)
Figure 2.15: Determination of fungal loads in mice tissue. The fungal burden in tissues from 3 mice from each group was determined by counting the CFU. The organs were
collected at the endpoints on day 21 for WT, 26 for complemented strain, and 60 for grx4 mutant.

2.3.3 A pilot transcriptomic analysis using RNA-seq provided further insight into role of Grx4 and also revealed complexities in the transcript structure of GRX4.

2.3.3.1 Grx4 is required for broad transcriptional remodeling in response to iron in C. neoformans

Fortuitously, I also had an opportunity to investigate the influence of Grx4 on transcriptional remodeling in response to iron levels in C. neoformans in a pilot RNA-Seq experiment offered by the NAPS Unit of the Michael Smith Laboratories. The NAPS unit needed RNA samples to test their methods for RNA-Seq library preparation and additionally offered complementary sequencing. I took advantage of this offer to add depth to the characterization of the grx4 mutant. Specifically, I wanted to further investigate the connection with iron homeostasis indicated by the aforementioned discovery of iron-related phenotypes (Figures 2.9-2.12) and the conserved role of Grxs in iron homeostasis in other fungi (N. Rouhier, et al., 2010). For this pilot experiment, the transcriptional profiles of the WT cells and the grx4 mutant were compared under iron-starved and iron overload conditions using RNA-Seq. Briefly, iron-starved cells were prepared for the WT strain and the grx4 mutant followed by growth for 6 h in LIM or LIM+Fe prior to RNA extraction (T. Lian, et al., 2005). It was previously shown that iron response becomes manifest within 6 hours See Materials and Methods, section 2.2.11, for the experimental set up). Due to the pilot nature of the experiment, RNA samples were prepared from only one biological replicate of the WT strain and the grx4 mutant to perform the transcriptome analyses (NAPS Unit, UBC).
Analysis of the RNA-Seq data was performed in collaboration with Dr. Daniel Croll using the R package cummeRbund ggplot2 version 0.9.3 (see Materials and Methods, 2.2.11). Although we did not have the biological and technical replicates to provide robust statistical confidence, we performed a preliminary analysis to identify differentially expressed genes (using a 3-fold cut off) in the grx4 mutant relative to the WT strain in LIM or LIM+Fe (Figure 2.16 and 2.17). Interestingly, differential transcript levels seen for genes such as the ferric reductase Fre 2, ferric-chelate reductase- Fre6, siderophore-iron transporter Str3, and siderophore iron transporter mirB identified by Quantitative RT-PCR (Figure 2.11) also showed up in the RNA-Seq data.

For the work in this thesis, we were able to analyze Gene Ontology (GO) terms in the RNA-seq data to generate an overview of the enriched functions among the differentially expressed genes in grx4 mutant compared to WT strain (GO terms available on locally installed Blast2GO were used as outlined in the Materials and Methods, section 2.2.11). As described below, the results from the GO term analysis revealed that the majority of differentially expressed genes in grx4 mutant were enriched for GO terms that particularly and notably were associated with iron sensing and the maintenance of iron homeostasis.

2.3.3.1.1 Categories of differentially expressed genes based on GO terms associated with molecular function in the grx4 mutant compared to WT cells grown under the iron-deplete condition.

We found that a total of 35 genes were down-regulated and 137 upregulated in the grx4 mutant in comparison with WT for cells grown under low-iron conditions. The GO term enrichment of these differentially-expressed genes revealed that the main molecular
functions affected by the absence of Grx4 under the iron-depleted condition included oxidoreductase activity (GO:0016491), Fe-S cluster binding (GO:0051536), and metal cluster binding (GO:0051540). The top 20 genes in these GO categories are listed in Appendix A: Table A2. Of note, the top-three enriched GO terms in the grx4 mutant were iron-consuming processes that were normally downregulated in the WT strain under iron-deplete conditions. This result suggests that lack of Grx4 might lead to loss of inhibition of the expression of iron-consuming functions during iron deficiency.
Figure 2.16: Enrichment of genes differentially expressed in RNA-Seq analysis of the *grx4* mutant and the WT strain under the iron-deplete condition. GO term enrichment was based on molecular function and visualized using the R package ggplot2.
2.3.3.1.2 Categories of differentially expressed genes based on GO terms associated with molecular function in the *grx4* mutant compared to WT cells grown under the iron-replete condition.

The analysis of the RNA-seq data from cells grown under the iron-replete condition revealed 17 genes with reduced transcript levels and 228 with increased transcript levels in the *grx4* mutant versus WT. The top three GO terms for these differentially expressed transcripts included oxidoreductase activity (GO:0016491), cation transporter activity (GO:00115101), and antioxidant activity (GO:0016209). The top 20 corresponding genes to the top three GO terms in the *grx4* mutant in the iron-replete condition are listed in Appendix A: Table A3. Interestingly, this list includes some of the iron acquisition genes such as the ferric reductases *FRE2* and *FRE6* and the siderophore transporters *STR3* and *MIRB* that previously showed upregulation in the *grx4* mutant compared to the WT in my qRT-PCR analysis under iron-replete condition (Figure 2.12). Of note, these iron acquisition genes are normally upregulated in WT under iron starvation. Overall, the results from the pilot RNA-seq analysis further supported the hypothesis that Grx4 is required for inhibition of iron-acquiring functions in *C. neoformans* in high iron conditions, and also contributes to maintenance of iron homeostasis in *C. neoformans*. 
Figure 2.17: Enrichment of genes differentially expressed in the RNA-Seq analysis of the *grx4* mutant and the WT strain in the iron-replete condition. GO term enrichment was based on molecular function and visualized using the R package ggplot2.
Furthermore, differentially regulated genes with similar and distinct patterns of expression in grx4 and cir1 mutants compared to the WT in iron-deplete and iron replete conditions were listed for future reference (Appendix A, Table A4- A7)

2.3.3.2 Analysis of the data from the pilot RNA-seq analysis identified several putative transcripts associated with the GRX4 locus.

During the analysis of the GRX4 deletion mutant, I realized that the annotation of the gene in the genome database (Broad) was modified with regard to intron-exon positions as part of the community effort to characterize and publish the genome of the WT strain H99 (G. Janbon et al., 2014). This change in the annotation prompted me to use the RNA-Seq data in hand to examine the experimental evidence for different isoforms from the GRX4 locus. For this analysis, the TopHat program (version 2.0.9) was used to align and to assemble the reads from the RNA-Seq analysis into transcripts, again in collaboration with Dr. D. Croll (Materials and Methods (section 2.2.11). The transcripts were then compared to the reference H99 C. neoformans serotype A genome annotation http://www.broadinstitute.org (Jan 2014) using Cufflinks 2.1.1, which was also used for further examination of transcriptomic data and de novo assembly of transcripts.

This analysis led to identification of three putative unannotated transcripts associated with the GRX4 locus in addition to the main transcript that I have used as the gene model for the work described in this thesis (Figure 2.18). De novo assembly of sequences showed that the three additional putative transcripts isoforms contain the conserved C-terminal Grx domain and variation among these transcript versions arises from differential inclusion of exons from the 5’ region of GRX4 locus. These results are
interesting because it is possible that the identified transcripts might encode variants of Grx4 with different functions, e.g., in binding proteins associated with Grx4. Of note, no isoform in our analysis was identical to the new GRX4 annotation available for the H99 genome provided by the Broad Institute (http://www.broadinstitute.org; January, 2014). This could possibly be due to differences in the conditions that were used in our RNA-seq analysis compared to those used in the studies that led to re-annotation of reference genome. Specifically, the conditions in our analysis were iron dependent whereas the conditions included in the published study included rich media (YPD), starvation medium (low glucose and low nitrogen medium), and pigeon guano broth. On the same note, a separate analysis performed by Dr. Croll on RNA-Seq data for the GRX4 transcripts obtained from available published RNA-seq libraries in different strain backgrounds and conditions also identified similar transcript variants (data not shown). The structures and expression patterns of the different transcript isoforms need to be independently validated in future experiments as a prelude to assessing their functional significance. Of note, the combined RNA-Seq analysis of the GRX4 region confirmed the existence of the transcript that was used for the studies reported here. Furthermore, this isoform encodes the polypeptide with the peptides that were experimentally identified in the initial mass spectrometry experiment outlined at the beginning of this chapter.
Figure 2.18: Exon–intron organisation of *C. neoformans* GRX4 locus. The organization according to (A) H99 database Jan 2014 (B) H99 database 2009 (C-F). RNA-seq data in this study is shown. The boxes represent the exons, whilst the dashed line represents introns. One isoform in our RNA-seq data (F) matches the Broad annotation in 2009 (B). No isoform is identical to the new Broad annotation (2014).

2.3.3.3 Analysis of GRX4 essentiality

2.3.3.3.1 Deletion of the entire GRX4 including the N-terminal region in a stable diploid (strain AI187) background shows that the N-terminal region of GRX4 is not essential.

The RNA-seq analysis of the *C. neoformans* transcriptome indicated the existence of different transcript isoforms from the *GRX4* locus. These isoforms mainly showed variation in expression of the N-terminal region of Grx4 and this led us to speculate that N-terminal region contributes to the biological role of Grx4 in *C. neoformans*. Given that the deletion mutant characterized to date retained the coding sequence for the N-terminal
region, I wanted to investigate the outcome of deletion of entire \textit{GRX4} locus. A construct was generated using a (NEO) marker linked to 5’ and 3’ flanking sequences of the entire \textit{GRX4} by three-step overlapping PCR (Figure 2.19 A), and the overlap PCR product was biolistically transformed into haploid H99 strain (See materials and methods 2.2.2.2). Surprisingly, numerous attempts to delete the entire locus in a haploid strain were unsuccessful.

The complexity of the \textit{GRX4} locus structure shown in RNA-seq data, and the failure to delete the entire locus including the N-terminal region, led me to test whether the N-terminal region is essential. For this approach, I employed a commonly used technique to identify essential genes in \textit{C. neoformans} and fungi in general, which is targeted gene replacement in a diploid strain with subsequent sporulation and phenotypic analysis of the resulting meiotic progeny (A. Idnurm, 2010). The deletion construct was biolistically transformed into the diploid strain Al187 (see section 2.2.2.2) and deletion of full-length \textit{GRX4} in the diploid H99 background was confirmed by PCR.

Positive transformants were subjected to sporulation (See Materials and Methods section 2.2.2.2.). Basidiospores were collected and incubated on YPD at 30°C and then transferred individually to YPD medium containing NEO (Figure 2.19 B). Spores that grew in presence of NEO were selected for further analysis. Deletion of \textit{GRX4} was first confirmed by PCR in the colonies that formed on YPD+ NEO (Figure 2.19 B), while colonies formed on YPD but not YPD+ NEO were shown to carry the WT copy of \textit{GRX4}. This was tested by PCR using primers that amplified an internal region within \textit{GRX4} to yield a fragment of 650 bp (Figure 2.19 A). As expected, colonies with deletion of \textit{GRX4}, displayed increased cell surface reductase activity in the presence of TTC (Figure 2.19 B). Overall,
these results show that sporulation of a stable diploid (strain AI187) generated a heterologous progeny population with respect to deletion of GRX4. Because spores with deletion of the entire GRX4 including the N-terminal region were viable, I concluded that the GRX4 N-terminal region is not essential in C. neoformans. The reason I was unsuccessful in deleting the entire locus including the N-terminal region in a haploid background remains to be investigated.
Figure 2.19: Full deletion of *GRX4* in a stable H99 diploid background. (A) *GRX4* is shown in dark blue arrow and neomycin knockout cassette replacing the entire *GRX4* locus is shown in red. (B) Spores were germinated on YPD and copied to YPD + 200μg/ml neomycin. Spores with deletion of *GRX4* grew in presence of NEO and formed red colonies.
in presence of TTC. Presence of the WT copy of GRX4 in Spores that only grew on YPD and not in presence of marker was confirmed by PCR.

2.3.3.3.2 Genetic segregation analysis of heterozygous auxotrophic markers in progeny isolated from a stable diploid with a confirmed deletion of GRX4

To confirm that meiosis had occurred during the generation of the basidiospores from the experiments described above in section 2.3.3.3.1, I undertook a more extensive genetic analysis of the collected spores. Specifically, I scored the progeny for the ade2 and ura5 genetic markers that were heterozygous in strain Al187 to confirm the expected single gene segregation of these markers in the spore population. The parent diploid strain Al187 was originally generated as a result of crosses between ade2 MATα x ura5 MATa strains (A. Idnurm, 2010). Basidiospores that were studied in section 2.3.3.3.1 above were transferred from YPD to YNB lacking adenine or uracil. Out of 52 colonies that were germinated on YPD, 29 (almost 55%) were ADE2 URA5 as shown by growth on both YNB-adenine and YNB-uracil, and 23 (44%) were ade2 ura5 as shown by no growth on either of the dropout plates (Figure 2.20). This analysis confirmed that meiotic reduction had indeed taken place. These results are consistent with previous results in the study done by Idnurm group where the sporulation event in diploid strain Al187 resulted in 77 total colonies of which 43 (56%) were ADE2 URA5, and 34 (47%) were ade2 ura5 (A. Idnurm, 2010). Overall, this extended genetic analysis confirmed that colonies studied in section 2.3.3.1 were the progeny produced by a meiotic event in strain Al187 upon sporulation.
Figure 2.20: Analysis of progeny. A total of 52 progeny on YPD were replica plated to media to test for the ADE2 and URA5 alleles.

2.4 Discussion

Understanding the mechanisms by which C. neoformans senses the mammalian host environment and regulates virulence factor expression is of great significance. Our group has previously shown that iron plays a central role in the pathogenesis of C. neoformans (W. H. Jung and J. W. Kronstad, 2011, W. H. Jung, et al., 2006, T. Lian, et al., 2005, S. Saikia et al., 2014).

Specifically, the GATA-type, zinc-finger protein Cir1 was previously identified as the main transcription factor that controls the iron regulon and expression of the known major virulence factors in C. neoformans (W. H. Jung, et al., 2006). Thus understanding the mechanisms by which Cir1 senses iron availability and contributes to proliferation of the fungus in mammalian hosts and identification of the key iron sources used by the fungus during infection are important goals. The components and features that contribute to Cir1 functions are not completely defined. To address this issue, proteins that are candidate
interaction partners with Cir1 were identified using a binding assay and a proteomic approach; one such protein was a monothiol glutaredoxin (Grx) with a signature CGFS active site.

Based on the conserved evolutionary functions of monothiol Grxs in binding iron-responsive transcription factors and regulation of iron homeostasis in other fungi, the work in this chapter was conducted to understand the role of monothiol glutaredoxin Grx4 in *C. neoformans*. I focused on determining the role of Grx4 by characterizing the phenotypic and transcriptional changes resulting from deletion of *GRX4*. I found that Grx4 contributes to the maintenance of iron homeostasis and has a critical role in the virulence of the fungus. Importantly, this is the first functional characterization of a monothiol Grx in a pathogenic fungus.

**2.4.1 The conserved C-terminal monothiol Grx domain of Grx4 binds to the N-terminal region of Cir1.**

*In silico* analysis indicated that CnGrx4 is a monothiol Grx with a highly conserved Grx domain. Studies in other fungi have revealed that monothiol Grxs inhibit iron-dependent transcription factors in response to both excess levels of iron and iron deficiency. These studies provide clues for the function of Grx4 in *C. neoformans*. For example, Aft1 in *S. cerevisiae* activates the expression of high-affinity iron transport functions in response to iron deficiency, and is inactivated by iron repletion (J. Kaplan, et al., 2006). It has been shown that Grx3 and Grx4 (Grx3/4) interact directly with Aft1, and several other cellular components, including GSH, and the Fra1 and Fra2 (Fe repressors of activation) proteins, to communicate information about cellular iron levels to Aft1 and
Inhibit its function upon iron repletion. Cells lacking Grx3/4 show constitutive expression of iron regulon genes (L. Ojeda, et al., 2006)

In the fission yeast S. pombe, which may be a closer evolutionary relative to C. neoformans, the transcription factor Fep1 (a Cir1 orthologue) is activated by excess iron accumulation and represses the expression of the genes involved in the acquisition of iron. In contrast, when S. pombe undergoes a transition from conditions of iron sufficiency to iron deficiency, the repressor activity of Fep1 needs to be shut down to allow for expression of high-affinity iron transporters (M. Jbel et al., 2009, B. Pelletier, et al., 2005). Of note, it has been shown that monothiol Grx4 is required for the iron-limitation-dependent inhibition of Fep1 under conditions of low iron (M. Jbel, et al., 2011).

In this study, I initially confirmed the binding between Grx4 and Cir1 in C. neoformans, which was originally identified by mass spectrometry analysis. I validated that monothiol Grx4 binds the N-terminal region of Cir1 as determined by ALPHAScreen analysis in vitro, and I was able to measure a dissociation constant of 2x10^{-6}M, which is indicative of a strong binding between Grx4 and Cir1. The recombinant full length Cir1 protein was however very susceptible to degradation, and I therefore purified a more stable, shorter MBP-fusion protein containing only the first 406 N-terminal amino acids (Figure 2.4). On this note, the full-length Cir1 polypeptide is 952 amino acids in length with a cys-rich domain (amino acids 162-189) and a conserved zinc-finger domain (amino acids 307-359) in the N-terminal region. It was previously shown that the N-terminal region (amino acids 1-406) of the protein was iron-responsive and responsible for the iron-dependent stability of Cir1 (W. H. Jung and J. W. Kronstad, 2011). The contribution of the C-terminal portion of the Cir1 has not been previously shown and remains to be
characterized. Taken together, my study defines the first example of a multidomain monothiol Grx4 as a binding partner of a major iron regulation transcription factor in the pathogenic fungus *C. neoformans*.

Defining the precise events underlining the binding between Cir1 and Grx4 needs further investigation, but what has been shown in *S. cerevisiae* and *S. pombe* can serve as a model of Cir1-Grx4 interaction in *C. neoformans*. The association of Grx3/Grx4 with Aft1 was shown to be independent of iron levels. In fact, Grx3/4 binding of Fe/S clusters serves as the iron sensor that communicates the cytosolic iron status to Aft1, and depletion of Grx3/4 leads to impairment of most iron-dependent processes, including formation of di-iron centers, and Fe-S cluster and heme biosynthesis. The Fe and/or Fe-S cluster-sensing role of glutaredoxins is also conserved in humans and other fungi that utilize Fe-responsive transcription factors (U. Muhlenhoff, et al., 2010).

In *S. pombe*, iron-dependent modulation of Fep1 activity is exerted by its interaction with the N-terminal DNA-binding of Grx4. The Fep1− Grx4 complex resides in the nucleus regardless of iron levels inside the cell. When iron is limited, Grx4 inhibits Fep1 function, which leads to dissociation of Fep1 from chromatin and derepression of the Fep1 regulon (M. Jbel, et al., 2011, K. D. Kim, et al., 2011, M. M. Molina-Navarro, et al., 2006, M. T. Rodriguez-Manzaneque et al., 2002). The involvement of Fe–S or Fe in this interaction was shown recently. Grx4 incorporates a glutathione-containing Fe-S cluster, and binds to iron-containing Fep1. Upon iron deprivation, the disassembly of the iron cluster promotes reverse metal transfer from Fep1 to Grx4, decreasing Fep1 interaction with promoters and de-repression of iron-import functions (J. Encinar del Dedo, et al.,
As mentioned, Fep1 (*S. pombe*) and Cir1 (*C. neoformans*) are orthologs and share significant amino acid sequence identity. Therefore, it is possible that these two proteins use similar mechanisms or partners in their interactions with monothiol Grxs.

It has also been shown that the monothiol Grxs are specific and efficient catalysts of deglutathionylation of their S-glutathionylated binding partners. Reversible glutathionylation is a prevalent form of cysteine modification and is recognized as a potential regulatory and a homeostatic protective mechanism that prevents cysteine oxidation to irreversible forms (C. L. Grek et al., 2013, M. D. Shelton and J. J. Mieyal, 2008). A large number of proteins have been identified as potentially regulated by reversible S-glutathionylation, including transcription factors such as p53 and NF-κB via conserved cysteines. This process affects their binding properties to DNA thereby regulating gene expression (J. J. Mieyal et al., 2008) (B. C. Liao et al., 2010) (C. S. Velu et al., 2007). It is therefore possible that Grx4 regulates the function of Cir1 through reversible S-glutathionylation, but this remains to be investigated.

### 2.4.2 The *grx4* mutant displays iron-related and virulence-related phenotypes

The original *grx4* mutant (from our collaborators Dr. Missall and Dr. Lodge) has the conserved C-terminal Grx domain replaced by a NATr cassette. I was able to replace the deleted region with the wild-type gene at the original locus to obtain a complemented strain (*grx4Δ::GRX4*). This strain was valuable in my examination of the role of the Grx domain in *GRX4* because it confirmed that the phenotypes of the deletion mutant were due to loss of Grx4. The phenotypic examination revealed that the association between Grx4 and Cir1 was reflected in the *grx4* mutant sharing iron-related and virulence-related phenotypes with the *cir1* mutant, including sensitivity to elevated levels of iron, increased
cell surface reductase activity and increased sensitivity to the pro-drug phleomycin. These results strongly suggest that \textit{grx4} mutant has dysregulated iron homeostasis and indicated that the association between Grx4 and Cir1 has functional significance in \textit{C. neoformans}.

Another piece of evidence that indicates dysregulated iron homeostasis in the \textit{grx4} mutant was elevation in transcripts for iron acquisition functions including the high-affinity iron uptake system (Cft1, Cfo1), siderophore transporters (Sit1, Str3, Mir1B) and ferric reductases (Fre2, Fre6) despite sufficient iron supply (Figure 2.11). The enhanced iron acquisition in the \textit{grx4} mutant in the presence of iron suggests that Grx4 has an inhibitory effect on iron acquisition in \textit{C. neoformans}, which can potentially lead to increased intracellular iron content. This can also explain the increased sensitivity of \textit{grx4} mutant to the pro-drug phleomycin compared to the WT. Phleomycin interacts with intracellular iron pools, causing the formation of ROS, which results in DNA damage and eventual cell death. Of note, sensitivity to phleomycin reflects the level of free intracellular iron (B. Pelletier, et al., 2002), (W. H. Jung, et al., 2006). Taken together these results support the role of Grx4 in maintenance of iron homeostasis in \textit{C. neoformans}. This notion is further supported by my observation that the \textit{grx4} mutant exhibited a growth defect in the presence of iron, which was restored by addition of exogenous levels of GSH (5mM). Given the known toxicity of free iron, unrestricted iron uptake and enhanced free iron in the cell might be the cause of the poor growth of \textit{grx4} mutant cells in high iron media. Iron plays a determinant role through the generation of oxidative stress due to its ability to catalyze the generation of hydroxyl radicals (OH\textsuperscript{-}) from H\textsubscript{2}O\textsubscript{2} in the Fenton reaction (J. A. Imlay, 2008, J. A. Imlay, et al., 1988, J. A. Imlay and S. Linn, 1988). The results shown above suggest that lack of Grx4 ultimately leads to increased levels of intracellular iron, which
may accelerate the Fenton reaction. This is further supported by the observation that addition of GSH ameliorated the growth defect of \textit{grx4} mutant in the presence of iron. This can be explained by antioxidant properties of GSH, including its role in regulating cellular iron homeostasis and scavenging and detoxifying iron ions, leading to protection against oxidative stress caused by iron toxicity (L. M. Milchak and J. Douglas Bricker, 2002). On this note, GSH depletion in both \textit{S. cerevisiae} and \textit{S. pombe} leads to loss of iron-dependent inhibition of Aft1/Aft2 and Fep1, presumably stemming from the requirement for GSH as a [2Fe-2S] cluster ligand in monothiol Grxs.

Alternatively, as described in section 1.5.1.1, GSH is a major component of the labile iron pool (LIP) of available iron in cells, and this adds significance to the role of GSH in iron homeostasis (R. C. Hider and X. L. Kong, 2011). In this regard, GSH acts as a major cytosolic ligand for Fe (II). Although the properties and features of this cytosolic pool are not completely understood, LIP is defined as the freely exchangeable iron that is loosely coordinated by water, small molecules, and GSH. Therefore, one can speculate that addition of GSH to the culture leads to restored growth of the \textit{grx4} mutant by chelating free excess iron and repression of iron acquisition components. Nevertheless, further studies are necessary to unravel the role of GSH in \textit{C. neoformans} both in association with Grx4 or independently.

\textbf{2.4.3 Grx4 has a central role in maintenance of iron homeostasis in \textit{C. neoformans}}

My preliminary RNA-seq analysis of the impact of the \textit{grx4} mutation further consolidated my findings on the role of Grx4 in maintenance of iron homeostasis. Recent genetic and biochemical analysis of monothiol Grxs have shown that their central role in the control of iron homeostasis (at the transcriptional and post-transcriptional levels) is
an important component of the cellular response to iron deprivation. I hypothesized that the positive and negative influence of monothiol Grxs on iron homeostasis may result from downstream regulators that transcriptionally or post-transcriptionally control the genes for iron homeostasis. To address this question, I performed RNA-seq analysis on the grx4 mutant and found that Grx4 is an important component of intracellular iron homeostasis.

In C. neoformans, iron starvation causes extensive transcriptional remodeling that is mediated by the transcription factors such as Cir1 and HapX to activate iron-and siderophore uptake pathways and repress iron-dependent and iron-consuming metabolic pathways such as heme biosynthesis, respiration and ribosome biogenesis during iron starvation (to spare iron). This system is reversed during iron sufficiency in order to avoid toxic effects.

In our RNA-seq data, I found that under the iron sufficient conditions, the transcripts for genes encoding the ferric reductases FRE2 and FRE6 and the siderophore transporters STR3 and MIRB (also designated SIT6) were highly upregulated compared to the WT. These genes previously showed upregulation in the grx4 mutant compared to the WT in my qRT-PCR analysis under iron-replete condition. Of note, these iron acquisition functions are normally upregulated in WT in response to iron starvation. My data from the RNA-seq analysis are also in agreement with my findings on the sensitivity of grx4 to elevated iron levels, as observed in my phenotypic assays and growth curves. On the same note, I found that DNA repair helicases and repair enzymes were highly upregulated in grx4 mutant particularly in the iron-replete condition and this is another clue leading to the conclusion that loss of Grx4 results in over accumulation of iron and subsequent DNA
In general, my RNA-Seq data support the hypothesis that Grx4 may constitute part of an “iron sensing complex” (possibly through binding iron or Fe-S clusters) that responds to alterations in iron levels and transmits signals for transcriptional activation though interaction with Cir1 or other binding partners. Furthermore, owing to the central metabolic role of iron, iron regulation and maintenance of iron homeostasis are interconnected with various other regulatory circuits in *C. neoformans* including pH regulation, gluconeogenesis, zinc metabolism, and oxidative stress response and many various metabolic pathways that are linked to iron homeostasis. Therefore, it is suggested that Grx4 affects both maintenance of iron homeostasis and linked pathways.

### 2.4.4 Grx4 is required for virulence in *C. neoformans*

As an important part of my current study, I could show Grx4 is also important for virulence in *C. neoformans*. Several studies have shown that pathogenic microbes link the availability of iron with virulence attributes (see 2.2.1) and my work shows a similar link between the iron homeostasis system and virulence via Grx4 and Cir1. Remarkably, I found that the *grx4* mutant lost the ability to elaborate capsule, produce melanin and grow at 37°C which is representative of human body condition. Importantly, deletion of *GRX4* leads to complete loss of virulence in inhalation model of infection in mice. I speculate that the lack of virulence of the *grx4* mutant most likely results from the combined defects in iron homeostasis in this mutant, since iron is critical for fundamental cellular processes (W. H. Jung and J. W. Kronstad, 2008, W. H. Jung, et al., 2006). The connections between
Grx4 functions and iron homeostasis can also occur at multiple levels, including the association with Cir1 and regulation of expression of the iron regulon, capsule formation, laccase, phospholipase, cell wall and membrane biosynthesis, in addition to signaling pathways (W. H. Jung, et al., 2006). Cir1 also regulates the expression of genes associated with iron uptake from heme (CIG1), siderophores (SIT1), and the production of melanin, in addition to other iron-related genes (W. H. Jung, et al., 2006). I found that the grx4 mutant also shares virulence-related phenotypes associated with the cir1 mutant. It has previously been shown that cir1 mutant has a defect in capsule and melanin production, as well as reduced growth at 37°C, and is also avirulent in a mouse model (W. H. Jung, et al., 2006). Overall, my study revealed that Grx4 binds Cir1 and subsequently influences iron homeostasis, transcriptional control, and the production of virulence-related factors in C. neoformans.

2.4.5 The N-terminal of Grx4 is not essential and exists in some but not transcript isoforms from the GRX4 locus

Re-analysis of the RNA-seq data led to the identification of three putative unannotated transcripts associated with the GRX4 locus, in addition to the main transcript that I have used as the gene model for the work described in this thesis (Figure 2.18). De novo assembly of sequences showed that these additional putative transcripts contain the conserved C-terminal Grx domain and variation among these transcript versions arises from differential inclusion of exons from the 5′ region of the GRX4 locus. Additionally the 2014 annotation of the GRX4 gene in the genome database (Broad) was modified with regard to intron-exon positions in the N-terminal of GRX4 as part of the community effort.
to characterize and publish the genome of the WT strain H99 (G. Janbon, et al., 2014). On this note, a notable characteristic of *C. neoformans* is the heterogeneity of genome sequences from different clinical and environmental isolates, and in many cases, this genetic variation results in altered expression patterns of genes (G. Hu, et al., 2011). Also, alternative splicing is common in *C. neoformans* and intron-dependent regulation of gene expression could play a major role in the biology of the fungus (R. Tarrio et al., 2008).

*C. neoformans* genes are intron-dense with an average of 5.7 per gene and the introns are generally small in size (median size: 56 nucleotides) (G. Janbon, et al., 2014). Also of significance is the fact that alternative splicing is a common feature for genes located on chromosome 3 (G. Janbon, et al., 2014) where GRX4 is also located. Another point that complicates the expression pattern of the *GRX4* locus is its antisense direction. The patterns of transcription on genes in antisense direction are regulated by growth conditions, suggesting a complex mechanism of gene expression regulation in these genes including *GRX4*. This prompted me to test the phenotypic outcome of deletion of the N-terminal region of the *GRX4* locus and investigate the role of this region in *C. neoformans*. However, despite numerous efforts, I was not able to acquire a deletion mutant when targeting this region and deleting the complete *GRX4* locus in the haploid strain H99. This led me to hypothesize that this region might be essential in *C. neoformans*, especially because it has previously been shown that the N-terminal region of GRX4 is essential for *in vivo* function in *S. cerevisiae* (B. Hoffmann et al., 2011, U. Muhlenhoff, et al., 2010). I tested this hypothesis by deleting the complete locus including the N-terminal region of *GRX4* in a diploid strain of *C. neoformans* by biolistic transformation. The results of this experiment showed that the meiotic progeny with deletion of *GRX4* were viable under normal growth
conditions, indicating that, unexpectedly, the N-terminal region of *GRX4* is not essential in a haploid background. At this point it is not clear why I was not able to delete the N-terminal region in the haploid strain H99. One possible explanation is that this region might be required for chromosome stability of the locus presumably because of an essential gene(s) in the locus (K. L. Ormerod and J. A. Fraser, 2013). On this note, N-terminal of *GRX4* locus is in close proximity to CNAG_02948, which encodes a peptide chain release factor 1. This is an essential gene and it shares a promoter with *GRX4* promoter, which might explain why I was not able to interfere with the N-terminal region in a haploid background. Combined, these data indicate a unique unusual arrangement of the *GRX4 locus in C neoformans*.

In summary of this study, I investigated the role of the monothiol Grx4 and obtained evidence that it binds a major iron transcription factor Cir1 and *grx4* and *cir1* mutants share similar iron-related and virulence related phenotypes. Investigating the mechanisms for iron homeostasis in *C. neoformans* will allow a better understanding of how *this pathogen* survives the environments encountered during infection and should provide additional potential targets for therapeutic intervention. My study on Grx4 underscores the significance of iron sensing in the expression of virulence factors leading to cryptococcosis. The knowledge of the role of iron in fungal infections has advanced immensely in recent years, and its potential application in treatment and diagnosis of fungal infections requires deeper and further insights.
Chapter 3: Analysis of the roles of glutathione synthetase in the AIDS-associated pathogen Cryptococcus neoformans

3.1 Introduction

The work presented in chapter 2 identified and characterized the role of Grx4 in iron homeostasis in C. neoformans. As described in section 2.3.2.2 I found that after 48 hours of iron starvation, the grx4 mutant had a growth defect in the presence of iron compared to WT (Figure 2.10). I speculated that grx4 mutant might be susceptible to oxidative stress upon iron starvation and/or iron repletion leading to impaired growth. On this note, I also found that the growth defect of the grx4 mutant was rescued by addition of exogenous GSH at concentration of 5 mM (Figure 2.11). This observation prompted me to further investigate the role of glutathione in C. neoformans. The experiments presented in this chapter of my thesis focused on examining the roles of GSH in iron homeostasis, cell wall integrity and expression of virulence factors in C. neoformans.

The tripeptide glutathione (GSH) or γ-glutamyl-cysteinyl-glycine (Figure 3.1) is the most abundant low-molecular-weight thiol in eukaryotic organisms and gram-negative bacteria (R. C. Fahey et al., 1978, H. Sies, 1999).
Figure 3.1: Structure of GSH: The tripeptide GSH consists of the amino acids glutamate, cysteine and glycine. A glutamyl amide linkage ligates glutamate and cysteine.

GSH serves in many biological processes including the synthesis of proteins and DNA, transport, enzyme activity, detoxification of heavy metals, xenobiotics and drugs, and defense against reactive oxygen species (ROS) (Figure 3.2) (A. Meister and M. E. Anderson, 1983). The “classic” role of GSH as thiol redox buffer and cofactor of antioxidant enzymes is particularly important because living organisms need to maintain an intracellular reducing environment (C. Mytilineou et al., 2002). GSH guarantees maintenance of the intracellular reducing environment and also acts as a cofactor for enzymes such as glutathione peroxidases and glutathione-S-transferases (GST) (K. Becker et al., 2003, A. Meister and M. E. Anderson, 1983). These enzymes are involved in protecting cells against reactive oxygen species (ROS), toxic metabolic intermediates and xenobiotics.

GSH is also known as a non-enzymatic antioxidant either alone or in conjunction
with vitamin C and vitamin E (C. L. Linster and E. Van Schaftingen, 2007). For example, it has been demonstrated that GSH and vitamin E protect against iron toxicity and inhibit iron-induced cell death in rat hepatocytes (L. M. Milchak and J. Douglas Bricker, 2002). It has also been shown that GSH and Vitamin C supplementation protects Arabidopsis seedlings from iron deficiency, preserves cell redox homeostasis and improves internal iron availability (L. Ramirez, et al., 2013). Intracellular glutathione also hinders the progression of heavy metal-initiated cell injuries by chelating and sequestering the metal ions in ectomycorrhizal fungi (M. Bellion et al., 2006)

Research on GSH has expanded rapidly in the last 10 years, and recent findings are also challenging the traditional view of GSH as the main player in redox control. In particular, recent studies present glutathione as a critical signaling molecule. For instance, it was recently shown that GSH activates the virulence of the intracellular pathogen Listeria monocytogenes (M. L. Reniere et al., 2015). Another fast-developing topic in GSH research is “protein glutathionylation” which has been suggested to have a major role in regulating the activity of metabolic and structural enzymes (P. Ghezzi et al., 2002) (D. A. Davis et al., 2003). Another critical role of GSH is in regulation of iron homeostasis (C. Kumar, et al., 2011, C. Berndt et al., 2014, C. H. Lillig and C. Berndt, 2013, C. E. Outten and A. N. Albetel, 2013, F. W. Outten, 2007, K. Sipos, et al., 2002). It has been shown that GSH is involved in iron metabolism by partnering with monothiol Grxs to assemble Fe–S clusters as described in section 1.5. GSH also serves as a major component of the labile iron pool (LIP) of available iron in cells, (R. C. Hider and X. L. Kong, 2011).

C. neoformans is exposed to numerous stresses during its life cycle in the hostile conditions of the human host, including nutrient deprivation, oxidative and nitrosative
stress, and high temperature. The defense antioxidant mechanisms that enable *C. neoformans* to survive these stress conditions include thioredoxins, catalases, peroxidases and glutathione peroxidases (Gpx), as well as the mitogen-activated protein kinase (HOG1) signal transduction and calcineurin/calmodulin pathways.

**Figure 3.2: Biological functions associated with GSH.** A partial list of GSH-dependent cellular pathways and proteins are illustrated.

In all GSH-containing species, GSH is synthesized de-novo by the sequential action of the γ-glutamylcysteine synthetase (γ-GCS) and GSH synthetase enzymes (Figure 3.3) These two enzymes are encoded by separate genes, *GSH1* and *GSH2*, in eukaryotes (*gshA* and *gshB*, respectively, in prokaryotes (S. C. Lu, 2009). The enzyme γ-GCS conjugates glutamic acid with cysteine and this is the rate-limiting step of GSH synthesis that is also
subject to feedback inhibition by GSH (A. Meister and M. E. Anderson, 1983). The gene encoding γ-GCS (GSH1) is essential in S. cerevisiae, S. pombe and C. albicans. Deletion of the genes encoding γ-GCS in these microorganisms leads to GSH autotrophy under normal growth conditions, indicating an essential role of (GSH1) in these fungi (B. Chaudhuri, et al., 1997) (Y. U. Baek, et al., 2004, C. M. Grant, et al., 1996, D. Wu, et al., 1994). Of note, it has been shown that GSH depletion causes an arrest of cell cycle at the G1 phase and cell death via apoptosis in S. cerevisiae and C. albicans (Y. U. Baek, et al., 2004, T. Russo et al., 1995, D. Spector et al., 2001). GSH1 is also essential in the model plant Arabidopsis thaliana, as lack of γ-glutamylcysteine synthetase abolishes root growth and cell division (T. Vernoux et al., 2000). In mammalian cells, mutation of GSH1 results in embryonic lethality, and blastocysts isolated from gsh1 mutant strain cannot grow without GSH. The exact reason why GSH is important for development and growth is still unknown (Z. Z. Shi et al., 2000).

In the second step, glutathione synthetase catalyzes the ATP-dependent synthesis of GSH by ligation of γ-glutamylcysteine (γ-Glu-Cys) with glycine. Genes encoding GSH synthetase have been identified in several bacterial and eukaryotic species (H. Gushima et al., 1983, N. Mutoh et al., 1991). It was shown that gsh2 deletion mutant in S. cerevisiae was viable but dependent on exogenous GSH for wild-type growth rates. It was also shown that the gsh2 mutant lacked GSH but accumulated the dipeptide γ-Glu-Cys, an intermediate in GSH biosynthesis. Of note, it has been shown that the γ-Glu-Cys intermediate was at least as good as GSH in protecting yeast cells against an oxidant challenge and served as an antioxidant and substitute for GSH in the cell; however, γ-Glu-Cys did not substitute for the essential function of GSH such as detoxification of harmful intermediates that are generated during normal cellular metabolism (C. M. Grant, et al., 1996)
Figure 3.3: Enzymatic pathway for GSH synthesis in eukaryotes. GSH is synthesized de-novo by the sequential action of γ-glutamylcysteine synthetase (γ-GCS) and GSH synthetase.

In spite of critical roles of glutathione in a plethora of biological processes, the roles of GSH and GSH biosynthesis in *C. neofomans* have not been explored. In the present study I generated mutants deficient in GSH by a targeted deletion of *GSH2* (CNAG_04647).
encoding glutathione synthetase. I then examined the role of GSH by detailed analysis of phenotypic outcomes manifested upon deletion of the GSH2 gene in *C. neoformans*.

### 3.2 Materials and methods

#### 3.2.1 In silico protein analysis

The amino acid sequence of GSH synthetase from the *C. neoformans* serotype A strain H99 ([http://www.broadinstitute.org/annotation-genome/cryptococcus_neoformans, 2014](http://www.broadinstitute.org/annotation-genome/cryptococcus_neoformans)) was used in a blastp search([http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins](http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins)) to identify orthologs in other fungal species and eukaryotes. Multiple sequence alignments were performed using Clustal Omega ([http://www.ebi.ac.uk/clustalO/](http://www.ebi.ac.uk/clustalO/)).

#### 3.2.2 Yeast strains, plasmids and growth media

The serotype A strain H99 of *C. neoformans* var. *grubii* was used as the WT strain along with three individual *gsh2* mutants, and the complemented strain *gsh2Δ::GSH2* (Table 3.1). The strains were maintained on YPD medium (1% yeast extract, 2% peptone, 2% dextrose and 2% agar). Selectable markers for transformation were from plasmids pJAF15 (hygromycin resistance) for mutants, and pJAF1 (neomycin resistance) for the complemented strain. Hygromycin was used for selection at 100μg ml⁻¹ and neomycin was used at 200μg ml⁻¹.

Low iron medium (LIM) was prepared as described previously (S. E. Vartivarian, et al., 1993) Briefly LIM was composed of 6.7g YNB plus 2% glucose in 1 L of iron-chelated H₂O using BIORAD chelex-100 and supplemented with the membrane impermeable iron chelator BPS. The pH was adjusted to 7.2 and the medium was filter sterilized. To prepare iron-replete media, hemin was added at 100 μM, or FeCl₃ was added at 10 μM or 100 μM final concentrations to low-iron medium. Cells were pre-cultured in YPD overnight at 30°C
and starved in LIM for 48 hours to make sure intracellular iron was depleted before harvesting and transferring cells to LIM or iron-replete media. Where indicated, 5 mM GSH dissolved in chelexed 25 mM Hepes buffer pH 7.4 was added to LIM cultures at 12 hours prior to harvesting the cells.

For spot assays on solid media, ten-fold serial dilutions of cells were spotted onto the agar media indicated in the text. Plates were incubated at 30°C for 2-3 days before being photographed.

**Table 3.1: Strains used in chapter 3**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Provided by/Prepared by</th>
</tr>
</thead>
<tbody>
<tr>
<td>H99</td>
<td><em>C. neoformans</em> wild-type strain</td>
<td>Dr. Joseph Heitman</td>
</tr>
<tr>
<td><em>gsh2Δ</em>₁ and <em>gsh2Δ</em>₂</td>
<td><em>gsh2</em> deletion mutants in H99 background</td>
<td>Dr. Guanggan Hu</td>
</tr>
<tr>
<td><em>gshΔ</em>₃</td>
<td><em>gsh2</em> deletion mutant in H99 background</td>
<td>Rodgoun Attarian</td>
</tr>
<tr>
<td><em>gsh2Δ::GSH2</em></td>
<td><em>GSH2</em> complemented strain in the H99 strain background</td>
<td>Rodgoun Attarian</td>
</tr>
</tbody>
</table>

**3.2.3 Generation of *gsh2* mutants and *gsh2Δ::GSH2* complemented strain**

Primers used for construction of all strains are listed in Table 3.2.

**Table 3.2: Primer sequences for strain construction**

<table>
<thead>
<tr>
<th>Allele constructed</th>
<th>Primer identification</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>gsh2:HYG</em></td>
<td>Gls1-1</td>
<td>GGGTGCAAGCTAATGTCACAA</td>
</tr>
<tr>
<td></td>
<td>Gls1-2</td>
<td>CATACATCCTCTTCTCCACGCTGCGAGGATGTGAG</td>
</tr>
<tr>
<td></td>
<td>Gls1-3</td>
<td>AGCTCACATCCTCGCAGGGGGAAGAGGAGGATGT</td>
</tr>
<tr>
<td></td>
<td>Gls1-4</td>
<td>TAGTTTCTACATCCTCTTACGGAGAAGGAGGATGT</td>
</tr>
</tbody>
</table>
Three individual \textit{gsh2} deletion mutants were constructed by homologous recombination using a hygromycin (\textit{HYG}) marker linked to 5' and 3' flanking sequences of \textit{GSH2} by three-step overlapping PCR (primer list 3.2). The overlap PCR product was biolistically transformed into the WT strain H99, and deletion was confirmed by PCR (Appendix, Figure B.2.). To reconstitute the deleted \textit{GSH2} in the mutant at the original locus, a genomic DNA fragment containing 1.1 Kb of upstream promoter region and a 1 Kb

<table>
<thead>
<tr>
<th>Allele constructed</th>
<th>Primer identification</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gls1-5</td>
<td>Glic1-5</td>
<td>GACACAGAAGAAGCACGGTCGAGAAGATGAGAAACTA</td>
</tr>
<tr>
<td>Gls1-6</td>
<td>Glic1-6</td>
<td>GCCAAGCAACACAGCAATGTAGA</td>
</tr>
<tr>
<td>Gls1-7NE</td>
<td>Glic1-7NE</td>
<td>GGATCGATTGTCAGAGGAGCA</td>
</tr>
<tr>
<td>Gls1-8NE</td>
<td>Glic1-8NE</td>
<td>TCGGTTGGGTAGTCGGTAGG</td>
</tr>
<tr>
<td>Gls1-9PO</td>
<td>Glic1-9PO</td>
<td>GCTCTTTCTGGATCCATCTAGT</td>
</tr>
<tr>
<td>Gls1-10PO</td>
<td>Glic1-10PO</td>
<td>CTGAACATCCTGTAAGGTCGT</td>
</tr>
<tr>
<td>\textit{gsh2\Delta::GSH2}</td>
<td>P9-PO</td>
<td>GCTCTTTCTGGATCCATCTAGT</td>
</tr>
<tr>
<td>P3C</td>
<td>CACTGCCGGCCTTACTATTTCTCTTGAAGAGCGCGTCCGAGT</td>
<td></td>
</tr>
<tr>
<td>P2C</td>
<td>ACGTGCCGACCGCTTTCTCAAGAGAACTAGTAACGGCGGCAGTG</td>
<td></td>
</tr>
<tr>
<td>P5C</td>
<td>GACGCGATTTTACGCACCTTTTAAGCTGCGAGATGGTAGGCT</td>
<td></td>
</tr>
<tr>
<td>P4C</td>
<td>AGCTCACATCCCGAGCTAAAGTGCGTGAAATCGCGTC</td>
<td></td>
</tr>
<tr>
<td>P6C</td>
<td>CACTAGGAGTACTCAGTAAGCGGTACATC</td>
<td></td>
</tr>
</tbody>
</table>

 Allele constructed Primer identification Primer sequence

Gls1-5 Glic1-5 GACACAGAAGAAGCACGGTCGAGAAGATGAGAAACTA
Gls1-6 Glic1-6 GCCAAGCAACACAGCAATGTAGA
Gls1-7NE Glic1-7NE GGATCGATTGTCAGAGGAGCA
Gls1-8NE Glic1-8NE TCGGTTGGGTAGTCGGTAGG
Gls1-9PO Glic1-9PO GCTCTTTCTGGATCCATCTAGT
Gls1-10PO Glic1-10PO CTGAACATCCTGTAAGGTCGT
\textit{gsh2\Delta::GSH2} P9-PO GCTCTTTCTGGATCCATCTAGT
P3C CACTGCCGGCCTTACTATTTCTCTTGAAGAGCGCGTCCGAGT
P2C ACGTGCCGACCGCTTTCTCAAGAGAACTAGTAACGGCGGCAGTG
P5C GACGCGATTTTACGCACCTTTTAAGCTGCGAGATGGTAGGCT
P4C AGCTCACATCCCGAGCTAAAGTGCGTGAAATCGCGTC
P6C CACTAGGAGTACTCAGTAAGCGGTACATC
downstream terminator region carrying the GSH2 gene was amplified by PCR. This PCR fragment was fused with the neomycin (NEO<sup>r</sup>) selectable marker (1.9 Kb) at its C terminus in an overlap PCR reaction. The overlap PCR product was introduced into the gsh2 mutant by biolistic transformation. Targeted integration was confirmed by PCR (Appendix, Figure B.2) and phenotypic assays.

3.2.4. Phenotypic and biochemical analysis

3.2.4.1. Growth Assay

The growth phenotypes of gsh2 mutants compared to the WT strain and the complemented strain gsh2Δ::GSH2 were tested in rich YPD and minimal YNB media. Briefly, strains were grown overnight in YPD or YNB, cells were harvested and serially diluted with sterile ddH2O. Serial dilution of cells starting at 10<sup>6</sup> were spotted on YPD or YNB agar medium and incubated at 30°C for 2 days.

Growth phenotypes of the strains was also determined in a parallel experiment where strains initially underwent iron-starvation for a period of 48 hours and were then plated on YPD, YNB, iron-deplete and iron-replete agar media as described in section 3.2.2. This experiment was also performed with introduction of glutathione to the culture (described in 3.2.2). Plates were incubated at 30° C for 2-3 days before being photographed.

3.2.4.2 Quantification of the intracellular content of total (reduced and oxidized or GSHt) glutathione

To determine the endogenous levels of glutathione in WT, gsh2 mutants and the complemented strain gsh2Δ::GSH2 cells were grown in YPD overnight and and the next day 2x10<sup>5</sup> cells were harvested and were re-introduced to fresh 50 ml YPD or 50 ml YNB, cells
were then harvested at 24 h and 48 h for the assay.

In parallel, the intracellular glutathione content of WT, gsh2 mutants and the complemented strain gsh2Δ::GSH2 was measured in an experimental set up where strains initially underwent 48 hours of iron starvation before the assay was performed. Then iron-starved cells (described in 3.2.2.) were re-introduced to fresh YNB with or without 5 mM GSH, and were harvested at 24 h and 48 h. The intracellular total glutathione levels (GSHt) were then quantified. Whole cells from each experimental set up were assayed for glutathione content using the BIOXYTECH® GSH/GSSG-412 colorimetric assay (OxisResearch; Portland, OR). This assay follows the principles of Tietze methods, which is an enzymatic method for quantitative determination of amounts of total (reduced and oxidized or GSht) glutathione (M. S. Anderson et al., 1996, O. W. Griffith, 1980, H. Guntherberg and I. Rademacher, 1966, H. Guntherberg and J. Rost, 1966, F. Tietze, 1969) This method also employs Ellman’s reagent (chromogen) 5,5'- dithiobis-2-nitrobenzoic acid or DTNB), which reacts with GSH to form a spectrophotometrically detectable product at 412 nm. In this assay, GSSG is determined by the reduction of GSSG to GSH, which is then determined by the reaction with Ellman’s reagent.

Here, to quantify reduced GSH, samples were obtained using a 5% cold metaphosphoric acid (MPA) extraction. The oxidized glutathione sample was obtained using the same type of extraction after scavenging the free thiols with scavenger 1-methyl-2-vinylpyridinium trifluoromethanesulphonate (M2VP). To quantify GSH, 10⁸ cells (in 1ml of culture) were harvested and were frozen at -70°C. To quantify GSSH, cells were first mixed gently with 200 μl M2VP in a microcentrifuge tube and were frozen at -70°C. After thawing, cells were resuspended in cold 5% MPA on ice (at 1/4 dilution of the volume of
original sample, GSSG samples were mixed with 300 μl 5% MPA, GSH samples were mixed with 250 μl 5% MPA) and were mixed with glass beads. Subsequently, the cells were homogenized by bead beating (3x 90sec, with 1min interval cooling on ice). After centrifugation at 4,500 rpm for 10 min at 4°C, 50 μl of the MPA extract was mixed with 700 μL of the assay buffer (Na·PO4) followed by addition of glutathione reductase, β-Nicotinamide adenine dinucleotide phosphate (NADPH) and the DTNB to each sample. The reaction mix was transferred to glass cuvettes and the change in color development of the chromogen DTNB during the reaction was measured by absorbance at 412 nm by spectrophotometer. The reaction was monitored for 5 minutes. In the blank reaction 50 μl of MPA was mixed with 700 μl of assay buffer only. The reduced and oxidized glutathione were calculated by measuring the change in absorbance at 412 nm as a linear function of the glutathione concentration in the sample. The concentration of GSH and GSSH in the sample was calculated by comparing with the rates of the standards (0.1, 0.25, 0.5, 1.5, 3 μM). Concentration of GSH and GSSG were calculated after subtracting the values generated by the blank reactions. The sum of quantified GSH and GSSG in each sample was calculated as total (reduced and oxidized or GSht) glutathione content. Statistical significance was determined using a single factor ANOVA variance test.

3.2.4.3 **Capsule formation, melanin production, and growth at 37°C**

Capsule formation was examined by differential interference microscopy (DIC). Briefly, cells were pre-grown in YPD, washed twice with low iron water (Li-H2O), diluted to 10⁶ cells/ml in Capsule Inducing Media (CIM), and grown for 24 hours at 30°C. CIM was prepared by dissolving 5g glucose, 5g asparagine, 4.78g HEPES, 0.4g K₂HPO₄, 0.25g CaCl₂·2H₂O, 0.08g MgSO₄·7H₂O, 1.85g NaHCO₃, add 1ml of 1000X salt stock solution in 1L
of Li-H$_2$O, pH adjusted to 7.2 using 1M MOPS made with low iron water, sterilized and 100 µl sterile thiamine (4 mg/ml) added. The cells were then resuspended in 1/10th volume of CIM, mixed 1:1 in India ink and visualized by negative staining on a Zeiss Axioplan 2 Imaging microscope by differential interference microscopy (DIC).

Melanin production was examined on l-3,4-dihydroxyphenylalanine (l-DOPA) agar containing 0.1% glucose. Cells were pre grown in YPD, washed once, counted, and adjusted to 2 × 10$^6$ cells ml$^{-1}$ in YPD. Serial dilutions were performed, and 5 µl of each dilution was spotted onto agar medium. Plates were incubated at 30°C.

The ability of the gsh2 mutants to grow at host temperature was tested by plating on solid media with subsequent incubation at 37°C.

3.2.4.4 Stress and drug response assays

Overnight pre-cultured cells of the wild-type, gsh2 mutants, and reconstituted gsh2Δ::GSH2 strains were washed, resuspended in H$_2$O, and adjusted to 2 × 10$^5$ cells/µl. The cell suspensions were serially diluted 10-fold, and 5 µl of each dilution was spotted onto YPD plates with or without 1.5 M KCl, 1.5 M NaCl, 0.5 mg/ml Congo Red, 1 mM diamide, 10 µg/ml fluconazole, 0.1 µg/ml miconazole, or 1 µg/ml amphotericin B. Plates were incubated for 3 to 5 days at 30°C and photographed.

3.2.4.5 Sensitivity to inhibitors of mitochondria electron transport chain

Overnight pre-cultured cells of the WT, gsh2 mutants, and reconstituted gsh2Δ::GSH2 strains were washed, resuspended in H$_2$O, and adjusted to 2 × 10$^5$ cells/µl. The cell suspensions were serially diluted 10-fold, and 5 µl of each dilution was spotted onto YPD plates supplemented with or without 75µM carbonyl cyanide m-chlorophenyl hydrazone (CCCP), 75 µg/ml Rotenone), 10mM potassium cyanide (KCN), 3 µg/ml
antimycin or 5 mM salicylhydroxamic acid (SHAM). Plates were incubated up to 5 days at 30°C and photographed.

3.3 Results

3.3.1 GSH synthetase encoded by GSH2 in C. neoformans belongs to the eukaryotic glutathione synthetase (eu-GS) family in the ATP-grasp superfamily.

The predicted glutathione synthetase encoded by GSH2 (CNAG_04647) in C neoformans had a length of 516 amino acids. BLAST analysis with this polypeptide sequence showed that GSH synthetase belongs to the Eukaryotic Glutathione Synthetase (eu-GS) family in the ATP-grasp superfamily. The members of this group of ATP-binding proteins cleave ATP to ADP and Pi, via the formation of an active intermediate y-L-glutamyl-L-cysteinyl phosphate (A. Meister and M. E. Anderson, 1983). An analysis of the amino acid sequence similarity of GSH synthetase in C. neoformans with other members of ATP-binding eu-GS family of proteins revealed a conserved GSH-binding domain, and ATP-binding domain, and a magnesium binding domain, in addition to a C-terminal glycine-rich loop and an alanine-rich segment. The C-terminal glycine-rich loop and an alanine-rich segment have with high degree of conservation and are proposed to be critical for the catalytic function of GSH synthetases (C. Fan et al., 1995) (Figure 3.4 A).

A Clustal Omega alignment of the amino acid sequence of the GSH synthetase of C. neoformans with four selected GSH synthetase sequences from other organisms revealed that the GSH synthetase protein sequence shares 37-39% identity with the four other eu-GS family gene products (39% amino acid sequence identity with GSH synthetase from S. pombe, 38% with GSH synthetase from S. cerevisiae, 39% with GSH synthetase from C. albicans, and 37% identity with GSH synthetase in Arabidopsis thaliana (Figure 3.4 B).
Figure 3.4: Domain structure and Blastp analysis of Gsh2 in *C. neoformans* (A) The identified domains underlined in GSH synthetase (*C. neoformans* var. grubii H99) are as follows: amino acids 16-51, dimerization site; 129-503, GSH binding pocket; 133-493, ATP-binding pocket; 147-401 magnesium binding site; 399-409, glycine-rich loop; 495-507, alanine-rich loop. (B) Percentage of identity in amino acid sequences in GSH synthetases across selected species as determined by Blastp analysis.
3.3.2 **GSH synthetase (Gsh2) is dispensable for growth under normal conditions in *C. neoformans***.

The growth of three individual *gsh2* mutants each with a total gene deletion was compared to growth of the WT and the complemented strain *gsh2Δ::GSH2* on YPD medium. The *gsh2* mutants were viable on rich YPD and minimal YNB media, indicating that *GSH2* is not an essential gene for growth under normal conditions for *C. neoformans*. This is in consistence with findings for Gsh2 in *S. cerevisiae*, which has also been shown to be dispensable for growth under normal conditions (C. M. Grant et al., 1997).

![Figure 3.5: Gsh2 is dispensable for growth under normal conditions in *C. neoformans*. Spot assays starting with 10^6 cells in the first spot and 10-fold dilutions in the spots to the right were plated on YPD or YNB agar.](image)

3.3.3 *gsh2* mutants are sensitive to iron starvation and require exogenous GSH for growth on minimal or iron-replete media after iron starvation

The growth phenotype of *gsh2* mutants post iron-starvation was tested in comparison with the WT strain and the complemented *gsh2Δ::GSH2* strain. Each strain was starved for iron in LIM for 48 hours and plated on LIM with 10μM FeCl₃, 100 μM FeCl₃ or 100 μM hemin (See materials and methods 3.2.4.1). All five strains including the *gsh2*
mutants displayed a robust growth phenotype on YPD plate after iron starvation. However, when plated on minimal YNB agar, gsh2 mutants displayed a prominent growth defect compared to the WT and gsh2Δ::GSH2 strains, indicating that the nutrient-limited condition of YNB did not support the growth of the gsh2 mutants after iron starvation. I speculated that this is likely due to lack of GSH levels in the gsh2 mutants that underwent iron-starvation. It is possible that the detrimental side effects of iron starvation are more pronounced in the presence of low levels of GSH in gsh2 mutants. Of note, it has been shown that GSH accumulates in the vacuole, and serves as a nitrogen and amino acid source during nitrogen starvation of S. cerevisiae (K. Mehdi and M. J. Penninckx, 1997).

Furthermore, as expected none of the strains grew on LIM agar plates (YNB+ 150 μM BPS). Testing the growth phenotype of the strains on LIM agar supplemented with iron showed that gsh2 mutants had a growth defect in the presence of 10 μM FeCl₃, 100 μM FeCl₃ or 100 μM hemin compared to the WT and gsh2Δ::GSH2 strains. These results led to speculation that a potential lack of GSH in gsh2 mutants might be underlying these observations as well. Therefore, I next sought to test the effect of exogenous levels of GSH on the observed phenotypes in gsh2 mutants. To test this, I pre-treated the gsh2 mutants, and the WT and gsh2Δ::GSH2 strains with exogenous GSH (5 mM). GSH was added to the cultures during the period that they were undergoing iron-starvation at 12 hours before harvest. Harvested cells were then plated on LIM plates + 10 μM FeCl₃, 100 μM FeCl₃ or 100 μM hemin. The results showed that addition of GSH recued the growth defect of the gsh2 mutants, as the mutants had a solid growth phenotype compared to the WT and gsh2Δ::GSH2 strains on YNB and iron-replete media (Figure 3.6).

These results reflect the importance of Gsh2 and ultimately GSH for growth under
conditions that are comparable to the conditions that *C. neoformans* may encounter in the human host. It is possible that lack of GSH in *gsh2* mutants makes them more susceptible to the effects of oxidative stress brought upon by iron starvation, and therefore addition of GSH restores the growth phenotype of *gsh2* mutants as a result of protective role of GSH against oxidative stress, or the auxotrophy of *gsh2* mutants for GSH itself. Also, given the proven role of GSH in sequestering iron and detoxification of metal ions it is likely that introduction of iron to *gsh2* mutants with significantly low levels of GSH leads to presence of unbound iron in the cell (M. Jozefczak et al., 2012). Free iron is known to have toxic effects on cellular metabolism (J. W. Eaton and M. Qian, 2002). Therefore addition of GSH to *gsh2* mutants potentially leads to chelating iron and reducing the amount of free metal inside cells, which ultimately results in undisturbed metabolism and solid growth in *gsh2* mutants in presence of iron.
Figure 3.6: Growth phenotypes of gsh2 mutants in YNB and iron-replete media after iron starvation. (A & B) Growth of strains on YPD, YNB, and LIM agar supplemented with iron sources. (C & D) Strains were pre-treated with 5 mM GSH and growth was evaluated on YPD, YNB, and LIM supplemented with different iron sources as shown.
3.3.4 The *gsh2* mutants have reduced intracellular total glutathione (GSHt) compared to the WT strain

The results of the growth assays in 3.3.3 suggested that the *gsh2* mutants had a decreased level of intracellular glutathione. I sought to examine this possibility by measuring intracellular levels of GSH in *gsh2* mutants grown under normal conditions without prior iron-starvation (Figure 3.7 A) or with prior iron-starvation (Figure 3.7 B) (See materials and method for details, section 3.2.4.2). This analysis revealed an average of 55 nmol of GSHt/mg of protein in the lysate from the WT cells, 45 nmol of GSHt/mg of protein in the lysate from the complemented strain cells, and an average of 5 nmol of GSHt/mg of protein in the lysate from the *gsh2* mutants cells grown in YPD. The GSHt content was slightly lower in strains grown in YNB compared to strains grown in YPD. This slight difference between GSHt levels calculated in YNB compared to YPD was not statistically significant. There was also a small decrease in GSHt levels in all strains at 48 h compared to 24 h, but this difference was not statistically significant. The 10X fold decrease in GSHt levels in *gsh2* mutants compared to the WT at both time points were statistically significant (*p* value<0.05). These results confirmed that *gsh2* mutants have lower GSH content compared to the WT strain and the complemented strain under normal growth conditions. Despite significantly low levels of GSH quantified in *gsh2* mutants in *C. neoformans*, these mutants are viable due to the potential accumulation of γ-Glu-Cys intermediate, which remains to be further confirmed. On this note, it was previously shown that the *gsh2* mutant lacked GSH but accumulated the dipeptide γ-Glu-Cys intermediate in GSH biosynthesis. Importantly, it has also been shown that the γ-Glu-Cys intermediate was at least as good as GSH in protecting yeast cells against an oxidant
challenge and served as an antioxidant and substitute for GSH in the cell (C. M. Grant, et al., 1997).

According to results outlined in section 3.3.3 and shown in Figure 3.6 of this chapter, I found that gsh2 mutants did not grow in YNB after 48 hours of starvation, but pre-treatment of the cells with 5 mM GSH restored growth of gsh2 mutants on YNB plates. Therefore, I sought to further validate that the addition of GSH resulted in restoration of growth in gsh2 mutants. To do this, strains were initially starved for 48 hours and were then grown in YNB or YNB supplemented with 5 mM GSH for 24 h and 48 h for GSht assay. The results from the GSht assay showed an average level of 60 nmol of GSht/mg of protein in the lysate from the WT cells pre-starved in iron and grown in YNB for 24 hours and 80 nmol of GSht/mg of protein in the lysate from WT cells pre-starved in iron, grown in YNB +5 mM GSH for 24 hours (1.3 fold higher) which was statistically significant. The results from this assay showed that the levels of GSht increased in gsh2 mutant after growth in YNB+ 5 mM GSH (compared to YNB) from 2.5 nmol of GSht/mg of protein in the lysate to 20 nmol of GSht/mg of lysate respectively (almost a 10-fold increase).

Overall, these findings lead to the conclusion that gsh2 mutants have a significantly lower amount of cellular glutathione, and addition of exogenous levels of GSH restores GSH content in these mutants. Lack of GSH levels in gsh2 mutants is most likely the underlying reason for the growth defect phenotype of gsh2 mutants after iron-starvation and introduction to YNB or ion-replete conditions.
**Figure 3.7: Total glutathione (GSht) levels in the gsh2 mutants.** (A) GSht levels in gsh2 mutants were measured and compared to the WT and the complemented strains grown in YPD (brown bars) or YNB (yellow bars). Samples were taken at 24 h and 48 h.
(Statistical analysis *p < 0.05). (B) Total glutathione (GSHT) levels in the gsh2 mutants were measured and compared to WT-H99 and the complemented strains that were iron starved and transferred to YNB (Black bar) or YNB+ 5 mM GSH (purple bars). Samples were taken at 24 h and 48 h. 3 biological replicates for each strain were prepared for each growth condition. (Statistical analysis was performed using using a single factor ANOVA variance test *p < 0.05 and *p < 0.0001

3.3.5 gsh2 is involved in multi-stress resistance

The role of GSH as the major cellular redox buffer is well established (K. Aquilano et al., 2014). Given the significance of GSH role in anti-oxidant defense, I sought to further examine the outcome of deletion of GSH2 and reduced GSH in C. neoformans, by phenotypic analysis of gsh2 mutants in presence of stressors. I found that gsh2 mutants were dispensable for C. neoformans growth in the presence of common ROS-generating stressors such as H2O2 (data not shown), however gsh2 mutants were in particular sensitive to diamide.

The growth defect of gsh2 mutants in presence of diamide are also consistent with my finding of low levels of GSHT in gsh2 mutants compared to the WT. Diamide is a thiol-oxidizing agent that mediates the direct oxidation of reduced glutathione (GSH) to GSSG (N. S. Kosower et al., 1969). Insufficient levels of GSH in gsh2 mutants can lead to loss of protection against toxicity of diamide. This potentially results in slower growth of gsh2 mutants compared to the WT and the complemented strain in presence of diamide (Figure 3.8).

The C. neoformans cell wall provides protection from the environment and determines the cell morphology during development of C. neoformans (S. R. Waterman et
al., 2007). To assess the role of Gsh2 in cell integrity, the WT strain, gsh2 mutants and the gsh2Δ::GSH2 strain were incubated on media containing Congo Red which is known to impair cell wall assembly, and to interfere with cell morphology. Additionally, resistance to salt stress has been correlated with C. neoformans survival in vivo, and genes involved and/or regulated by the HOG pathway are expressed during osmotic stress (Y. J. Ko et al., 2009). To test these stress phenotypes, the WT strain, the gsh2 mutants and the gsh2Δ::GSH2 strain were incubated on media containing high salt concentrations. The gsh2 mutants displayed a marked sensitivity to both Congo Red and high salt concentrations (Figure 3.8). This information suggests that Gsh2 is required for assembly of cell wall or expression of genes for cell wall synthesis. Furthermore, the confirmed lack of GSHT in gsh2 mutants may also account for an overall higher oxidative state of the cells.

![Figure 3.8: The gsh2 mutants have increased susceptibility to salt stress and thiol oxidizing agent diamide.](image)

Tenfold serial dilution of cells of the indicated strains were spotted onto solid YPD without or with 1.5 M NaCl, 1.5 M KCl, or 1mM diamide. The plates were incubated at 30°C
3.3.6 Lack of Gsh2 affects virulence factors in *C. neoformans*

Growth at human body temperature, production of melanin, and capsule are classical virulence factors for *C. neoformans*. For example, it has been shown that melanized cells are more resistant to ROS and reactive nitrogen intermediates (RNI) than non-melanized cells (Y. Wang and A. Casadevall, 1994). Strains that do not produce melanin are reported to have attenuated virulence in animal models (K. J. Kwon-Chung and J. C. Rhodes, 1986). I tested the expression of these major virulence traits in *gsh2* mutants and compared the resulting phenotypes to those in the WT and reconstituted strains. I found that that *gsh2* mutants did not produce capsule or melanin, and lost the ability to grow at 37°C. These findings indicate that an intact GSH biosynthesis pathway and sufficient levels of GSH are required for the elaboration of virulence traits in *C. neoformans*. 
Figure 3.9: The gsh2 mutants are impaired in virulence-related traits. Loss of GSH2 leads to virulence-related phenotypes including loss of capsule formation, an altered melanin production, and poor growth at 37°C.

3.3.7 gsh2 mutants display increased susceptibility to antifungal drugs

The roles of GSH in the detoxification of drug compounds and resistance against antibiotics have been described in Cyanobacteria, *E. coli*, and *C. albicans* (J. C. Cameron and H. B. Pakrasi, 2011, M. Kominkova et al., 2015, B. Maras et al., 2014). For instance, it has been shown that GSH metabolism plays an important role in the global organization of *C. albicans* cells for resistance to fluconazole (B. Maras, et al., 2014).

This led me to hypothesize that gsh2 mutants in *C. neoformans* will have an
impaired protection against antifungal challenge, given their significantly low levels of GSH. I tested this hypothesis by phenotypic analysis of gsh2 mutants in presence of the antifungal drugs that are currently used to treat cryptococcosis. Compared to the WT and the complemented strain, the gsh2 mutants displayed increased sensitivity to theazole drugs including fluconazole and miconazole, which are known to target and inhibit ergosterol biosynthesis. The gsh2 mutants displayed sensitivity to amphotericin, which binds irreversibly to ergosterol, resulting in disruption of membrane integrity. Taken together results support the idea that Gsh2 and cellular GSH alter susceptibility of C. neoformans to antifungal drugs.

Figure 3.10: Deletion of GSH2 Increases susceptibility to antifungal agents. The growth of strains in media containing the antifungal drugs fluconazole, miconazole and amphotericin B is shown. Ten-fold serial dilutions of cells (starting at $10^6$ cells) were spotted onto YPD plates with and without the antifungal drug indicated. Plates were incubated at 30°C for 3 days.
3.3.8 *gsh2* mutants display subtle susceptibility to mitochondria inhibitors

It is known that GSH is synthesized in the cytosol and transported into the mitochondria via an energy-dependent transporter (A. Meister, 1988a, b). GSH is important for the maintenance of optimum mitochondrial function. Distinguished from cytosolic GSH, mitochondria also contain GSH (mGSh) and it has also been suggested that decreases in cellular GSH availability in the mammalian cells promote mitochondrial damage via increased ROS (A. Jain et al., 1991).

This led to the hypothesis that loss of GSH2 in *C. neoformans* and lack of synthesis of GSH in these mutants will affect mitochondrial functions. I tested this hypothesis by phenotypic analysis of *gsh2* mutants along with the WT and the complemented strain in presence of inhibitors of mitochondrial functions, including 75μM CCCP, 75 μg/ml rotenone, 10 mM (KCN), 3 μg/ml antimycin or 5 mM SHAM. Results show that *gsh2* mutants have a subtle sensitivity compare to the WT and the complemented strain to respiratory chain enzymes inhibitors. These results suggest that GSH2 and cellular levels of GSH influence mitochondrial functions. The role of mGSH in *C. neoformans* remains to be investigated.

![Figure 3.11: gsh2 mutants display a subtle susceptibility to ETC inhibitors.](image)

The growth of strains in media containing the inhibitors of mitochondrial function is shown.
Ten-fold serial dilutions of cells (starting at $10^6$ cells) were spotted onto YPD plates with and without the compounds indicated. Plates were incubated at 30°C for 5 days.

### 3.4 Discussion


The biosynthesis of GSH has been studied in a variety of organisms such as *E. coli*, *S. cerevisiae*, *C. albicans* and *Trypanosoma brucei* (S. Bouter et al., 1988, J. C. Lee et al., 2001, Y. U. Baek, et al., 2004, T. T. Huynh et al., 2003). It was shown that GSH biosynthesis is not essential in these microorganisms as long as they are supplemented with an exogenous high concentration of GSH in the extracellular environment. In contrast, glutathione synthetase deletion mutants in *S. cerevisiae* do not require exogenous GSH for growth, as they accumulate and utilize the GSH precursor γ-glutamylcysteine (γ-Glu-Cys), as a substitute under normal growth conditions (C. M. Grant, et al., 1997).

In *C. neoformans*, glutathione metabolism genes were induced during heat shock, nitrosative stress or during phagocytosis by macrophages (T. A. Missall et al., 2006). Interestingly, glutathione reductase (Glr1), which reduces the disulphide form of glutathione (GSSG) to GSH, has been shown to be important for virulence in *C. neoformans*. 
as a *glr1* deletion mutant was found to be avirulent, and was more sensitive to nitrosative stress and macrophage killing (T. A. Missall, et al., 2006). This further highlights the importance of glutathione biosynthesis and metabolism in *C. neoformans*. However, the role of glutathione in *C. neoformans* has not been explored so far. In this chapter, I characterized the role of *GSH2* gene, which encodes GSH synthetase in the glutathione biosynthesis pathway, and I uncovered a pleiotropic consequence of lack of GSH due to deletion of *GSH2* in *C. neoformans*.

### 3.4.1 GSH synthetase is dispensable for growth under normal growth conditions

A total gene deletion of *GSH2* in *C. neoformans* was generated by replacement of the entire *GSH2* open reading frame with *HYG* gene. Despite significantly low amount of GSHt measured in *gsh2* mutants, they were viable on rich YPD and minimal YNB without exogenous glutathione. This indicated that *GSH2* is not an essential gene for normal growth under non-stressed conditions. My findings were in agreement with findings on *Gsh2* in *S. cerevisiae*. It was shown that *Gsh2* was dispensable for growth under normal conditions in *S. cerevisiae* due to accumulation of the dipeptide γ-Glu-Cys intermediate. This intermediate is the product of *GSH1* and was shown to protect yeast cells against an oxidant challenge and served as an antioxidant and substitute for GSH in the cell (C. M. Grant, et al., 1997). Similarly, I predict that accumulation of the γ-Glu-Cys intermediate in *gsh2* mutants in *C. neoformans* is sufficient for growth in YPD and YNB. This prediction will require further studies for confirmation.
3.4.2 Phenotypic analysis of gsh2 mutants suggest an interplay between GSH homeostasis and iron homeostasis in C. neoformans

The growth phenotypes of gsh2 mutants subjected to iron starvation revealed a further understanding of the role of Gsh2 in C. neoformans. I found that iron-starved gsh2 mutants had a normal growth phenotype similar to the WT and the complemented strains when plated on rich YPD agar, but showed a pronounced growth defect when plated on minimal YNB agar. One potential explanation for the observed difference is that compared to YNB, YPD has a higher content of amino acids including methionine, cysteine and their constituent sulfur. Sulfur also serves as a constituent of organic molecules like coenzyme-A, GSH and Fe-S clusters. The importance of sulfur in fungal species such as C. albicans, C. glabrata and Aspergillus fumigatus has been demonstrated (Marzluf et al., 1997). I speculate that iron depletion in gsh2 mutants in the absence of GSH may lead to a higher nutritional demand for growth in gsh2 mutants, which is satisfied in the nutrient-rich condition of YPD compared to YNB. Importantly, addition of iron to YNB did not restore growth in gsh2 mutants, but pre-treatment of gsh2 mutants with GSH led to restoration of growth on YNB. Overall, these results clearly indicated that depletion of iron in gsh2 mutants led to auxotrophy for GSH. I also measured GSht content in cells grown in YNB after undergoing iron starvation. I first found that iron-starved gsh2 mutants had slightly a lower amount of GSht compared to the non-starved cells and, importantly, pre-treatment of gsh2 mutants with GSH led to a significant increase in GSht levels.

GSH requirement for viability of gsh2 mutants in C. neoformans in iron-starvation conditions can theoretically be linked to antioxidant properties of GSH in this situation. It has previously been shown that GSH treatment in A. thaliana prevented the accumulation
of ROS induced by iron deficiency (L. Ramirez, et al., 2013). It is also known that iron is a constituent of several components of the electron transport chain in mitochondria and thus iron deficiency disrupts normal electron transfer resulting in the overproduction of ROS (M. Graziano and L. Lamattina, 2005, W. H. Tong and T. A. Rouault, 2006) (L. Ramirez, et al., 2013). Under these conditions, the high levels of ROS generated exceed the control capacity of the antioxidant system, causing cell oxidative damage. It is known that the decrease in GSHt content during iron deficiency could negatively affect the cell redox homeostasis, and addition of the exogenous of GSH controls the redox imbalance generated from an excess of ROS production under iron-deficient conditions (L. Ramirez, et al., 2013). Alternatively, GRXs can modulate protein activity by glutathionylation, a reversible post-translational modification involving disulfide formation between a protein thiol and GSH. This modification occurs more frequently in response to increased ROS, and protects the proteins from irreversible oxidation (I. Dalle-Donne et al., 2008). So it is possible that very low levels of GSH in gsh2 mutants renders GSH-dependent proteins such as glutaredoxin (Grx4), glutathione-S-transferases (GSTs), and glutathione peroxides (GPXs) in C. neoformans, unable to make use of GSH as a cofactor. Consequently, this would result in an imbalance of the intracellular redox homeostasis and impairment of cellular functions dependent on these enzymes. Nevertheless, further studies are required to determine whether specific molecular mechanisms triggered by GSH could be occurring to preserve cellular homeostasis during iron deficiency in C. neoformans.

The GSH requirement for viability of gsh2 mutants after iron starvation could also be theoretically linked to iron-metabolism and iron-sensing functions of GSH (H. R. Lopez-Mirabal et al., 2007) (C. Kumar, et al., 2011) (H. R. Lopez-Mirabal, et al., 2007). The role of
GSH for sensing iron is conserved between *S. cerevisiae* and *S. pombe*. It has been shown that regulating gene expression as a function of iron availability and sensing the iron inhibitory signals in *C. neoformans* share common components and iron-responsive transduction pathways with *S. cerevisiae* and *S. pombe* (D. J. Kosman, 2003). Therefore it is possible that iron starved *gsh2* mutants are auxotrophic for GSH because GSH is required for iron sensing (H. Li and C. E. Outten, 2012), which further explains why addition of iron to YNB, did not restore growth of *gsh2* mutants. It is also possible that GSH is required for iron-dependent regulation of Cir1, or other transcription factors in iron network of *C. neoformans*, including HapX. These predictions need to be further investigated. Another possible aspect is that GSH depletion is shown to cause induction of an iron starvation-like response that impairs cytoplasmic Fe–S protein maturation in yeast species due to the role of GSH and Grx partners in stabilizing [2Fe-2S] groups (H. Li and C. E. Outten, 2012, J. C. Rutherford, et al., 2005) (K. Sipos, et al., 2002), (S. Bandyopadhyay, et al., 2008a).

GSH also plays a critical role in stabilizing the labile iron pool that supplies iron for the synthesis of heme or [Fe-S] clusters (R. C. Hider and X. L. Kong, 2011). It is possible that lack of GSH in *gsh2* mutants may lead to the presence of unbound iron in *gsh2* mutants, which is not bioavailable for growth. Therefore addition of GSH to *gsh2* mutants results in binding and stabilizing iron, which becomes bioavailable to support growth of *gsh2* mutants.

Overall, these findings suggest that an inadequate supply of GSH in *gsh2* mutants in the absence of endogenous biosynthesis likely affects the ability of the *C. neoformans* to survive the harsh iron- and nutrient-restricted conditions of the host. I speculate that this is possibly due to the role of GSH in iron homeostasis in *C. neoformans.*
Taken together, my results suggest that GSH plays a central role under iron deficiency-triggered stress conditions, protects *C. neoformans* from iron deficiency, preserves cell redox homeostasis and is required for internal iron availability. This suggests that specific depletion of the glutathione in *C. neoformans* would impinge on its survival in the host, also raising the possibility of targeting GSH synthesis pathway to treat cryptococcal infections. Interestingly, an antifungal compound from garlic, diallyl sulphide, and the protein kinase inhibitor staurosporine, have both been shown to cause their antifungal activity by depletion of glutathione in *C. albicans* (S. Yousuf et al., 2011) (K. M. Lemar et al., 2007).

### 3.4.3 Phenotypic characterization of *gsh2* mutants revealed that Gsh2 is required for a plethora of functions in *C. neoformans*

As a key discovery of the phenotypic analyses of *gsh2* mutants, I found that Gsh2 has pleiotropic functions in *C. neoformans* including the response to diverse antifungal drugs including azoles and polyene drugs, cell wall integrity, salt stress responses, and virulence factor production. Of note, the pleotropic role of GSH in eukaryotes is known, and given the very low levels of GSHt in in *gsh2* mutants, elaboration of several phenotypes is expected.

In this study, I found that *gsh2* mutants exhibited sensitivity to external stressors such as high temperature, high salts, and antifungal agents. The ability to sense and respond to a hostile host environment is a crucial element for virulence of *C. neoformans*. Various extracellular or intracellular stresses such as high temperature, high salt, and antifungal drugs lead to accumulation of misfolded or damaged proteins inside the cell, and cause cell damage that is prevented by their removal through several response
mechanisms in *C. neoformans*. These responses are evoked and coordinated by diverse signaling cascades, including the stress-activated HOG pathway, PKC1/MAPK, and Ca\(^{2+}\)/calcineurin pathways. These signaling pathways are further regulated by the ubiquitin-proteasome system (C. S. Hemenway and J. Heitman, 1999) (Y. S. Bahn et al., 2005, K. W. Jung et al., 2012, K. T. Lee et al., 2014) (Y. J. Ko, et al., 2009) (K. W. Jung and Y. S. Bahn, 2009) (T. B. Liu et al., 2011) (Y. S. Bahn and K. W. Jung, 2013). My findings suggest the presence of a crosstalk between GSH homeostasis and these response pathways in *C. neoformans*.

In *C. neoformans*, *gsh2* mutants were sensitized to salt stress (1.5M NaCl and KCl). Similar effects have been reported in plants (*A. thaliana, Brassica napus, and Solanum lycopersicum*) where GSH protects against exogenous osmotic stress (J. H. Chen et al., 2012, V. Mittova et al., 2003) (J. M. Ruiz and E. Blumwald, 2002). The salinity stress in plants leads to excessive generation of ROS, which results from impaired electron transport processes in chloroplast and mitochondria as well as photorespiration. Resistance to salt stress is usually correlated with GSH acting as an antioxidant. My results suggest that GSH might have a role in mitigating salt-induced oxidative stress in *C. neoformans*.

I also found that *gsh2* mutants displayed increased susceptibility to major antifungal drugs including amphotericin B, fluconazole and miconazole compared to the WT and complemented strains. These drugs are commonly used to treat cryptococcal meningitis. Previously, the role of GSH in resistance to antifungal drugs has been demonstrated in *C. albicans*, and it was shown that increased levels of GSH lead to a more efficient elimination of fluconazole from the cells (B. Maras, et al., 2014). This could be due to the role of GSH in detoxification of drugs and xenobiotics or in ergosterol biosynthesis.
pathway. In this context, it has been shown that depletion of GSH leads to an iron starvation–like response and repression of ergosterol biosynthetic enzymes in yeast (C. Kumar, et al., 2011). Furthermore, gsh2 mutants display a subtle susceptibility to 75 μM CCCP, 75 μg/ml rotenone, 10mM KCN, 3 μg/ml antimycin or 5 mM SHAM, compared to the WT and complemented strains. This finding suggests that the role of Gsh2 in GSH production may have an impact on mitochondrial functions in C. neoformans. It has been shown that depletion of GSH leads to repression of genes encoding Fe–S and heme proteins of mitochondrial respiration, and the tricarboxylic acid cycle in yeast (C. Kumar, et al., 2011). Of note, despite its exclusive synthesis in the cytosol, GSH is distributed in intracellular organelles including the endoplasmic reticulum (ER), nucleus, and mitochondria. In mitochondria, however, GSH represents a minor fraction of the total GSH pool (10–15%) (M. Mari et al., 2009). Given the volume of the mitochondrial matrix, the concentration of mitochondrial GSH (mGSH) is similar to that of cytosol (10–14 mM) (M. Mari, et al., 2009) (C. Garcia-Ruiz et al., 1992) (O. W. Griffith and A. Meister, 1985). Therefore, it is possible to speculate that lack of intracellular GSH in gsh2 mutants may affect the mGSH levels and impair mGSH functions. mGSh protects mitochondrial functions in oxidative phosphorylation and ATP production and maintains mitochondria genome stability(M. Mari, et al., 2009). The importance of mitochondrial functions has been demonstrated in other cryptococcal studies, in which deletion of a mitochondrial gene encoding the alternative oxidase of mitochondria resulted in defects in tolerance to the stress environment in macrophages and attenuation in cryptococcal virulence (S. Akhter et al., 2003). Importantly, gsh2 mutants also displayed enhanced sensitivity to cell wall damaging agent Congo red. This suggests that GSH might serve as a signalling component.
in regulation of actin cytoskeletal architecture and cell wall integrity in *C. neoformans*. In this context, cell wall biogenesis, composition and the maintenance of cell integrity in *C. neoformans* is regulated by the protein kinase C (*PKC1*) signal transduction pathway (K. J. Gerik et al., 2008). It has been shown that GSH can antagonize PKC isozymes in mammalian cells. There is evidence that glutathione inhibits PKC isozymes by a yet unknown non-redox mechanism. Also, depletion of the intracellular GSH pool is proposed to lead to loss of a negative regulatory mechanism over PKC (N. E. Ward et al., 1998). Whether or how *PKC1* is regulated by glutathione in *C. neoformans* is not known and remains to be investigated.

I also found that *gsh2* mutants did not express classical virulence traits in *C. neoformans* including capsule production, melanin formation and growth at 37°C. Capsule production is a complex and dynamic process that includes intracellular polysaccharide synthesis, secretion of capsular polysaccharide via vesicles, and extracellular assembly (A. Yoneda and T. L. Doering, 2006). Cell wall components such as α-1,3-glucan and chitin-derived molecules are also required for GXM (major capsule component) anchoring to the cell surface and cell wall integrity contributes to formation of capsule (T. L. Doering, 2009). Elaboration of virulence-related phenotypes in *gsh2* mutants suggest that Gsh2 is potentially required for the establishment of the proper capsule or cell wall structure required for anchoring of capsule polysaccharides to α-1,3-glucan at the cell surface. It is also possible that Gsh2 via GSH is required for proper trafficking of materials for capsule production. Similarly, loss of Gsh2 may interrupt the processing and/or transport of laccase, resulting in loss of melanization. One of the key factors in the virulence of *C. neoformans* is the ability to grow at human body temperature (P. R. Kraus,
et al., 2004). In the present investigation, I speculate that defective growth of gsh2 mutants at 37°C is due to lack of GSH in these mutants. On this note, it has been shown that intracellular GSH levels are elevated during heat shock in mammalian cells and depletion of GSH leads to thermal sensitivity in mammalian cells and maize roots (J. B. Mitchell et al., 1983) (J. Nieto-Sotelo and T. H. Ho, 1986)). Emphasizing on the role of GSH in virulence, it is interesting to add that in a recent study reported by Reniere, et al., it was shown that glutathione activates virulence gene expression of intracellular bacterial pathogen Listeria monocytogenes (M. L. Reniere, et al., 2015). The intracellular lifecycle of L. monocytogenes is entirely dependent on the thermoregulator PrfA. PrfA is a member of the cAMP -Fnr family of transcription factors and is referred to as the master regulator of virulence as it directly regulates the transcription of nine virulence factors in L. monocytogenes (N. E. Freitag et al., 2009). In the report by Reniere et al., it was shown that PrfA senses the glutathione concentration to regulate its biphasic lifestyle and the switch from saprophyte to pathogen, and lack of GSH leads to complete loss of virulence in L. monocytogenes (M. L. Reniere, et al., 2015). Overall, it is possible that GSH has conserved and similar activating roles in virulence gene expression in other intracellular pathogens such as C. neoformans. Given the many faces and roles of GSH, it is predicted that lack of sufficient GSH in gsh2 mutants would impair virulence in C. neoformans as a result of combined defects of gsh2 mutants outlined in this thesis, including loss of capsule production, impaired melanization and loss of ability to grow at 37°C. Experimental infections in mice could be used to investigate this prediction.
Chapter 4: Discussion

4.1 Iron Homeostasis in *C. neoformans*

The research presented in this thesis provides further understanding of mechanisms of iron homeostasis in *C. neoformans*. Iron homeostasis is directly related to virulence of AIDS-associated fungal pathogen *C. neoformans*, as iron regulates the elaboration of major virulence factors in *C. neoformans* including the pigment melanin and the polysaccharide capsule (E. S. Jacobson and J. D. Hong, 1997) (K. L. Tangen, et al., 2007) (W. H. Jung and J. W. Kronstad, 2008). Therefore, identifying the genes and gene products that contribute to the iron regulatory network is essential to understanding the pathogenesis of *C. neoformans*. Several transcription factors in *C. neoformans* regulate iron homeostasis and other aspects of adaptation to the host environment (J. Kronstad et al., 2012). Our group has previously shown that Cir1 is the master iron regulator that influences the expression of iron and virulence-related genes including genes associated with iron uptake from heme (*CIG1*), a siderophore transporter (*SIT1*), and the production of melanin (*LAC1*). Cir1 functions also as an activator for growth at 37°C (host temperature) which is an important virulence trait in *C. neoformans* (W. H. Jung, et al., 2006). The mechanisms by which *C. neoformans* senses the mammalian host environment and regulates virulence factor expression are promising areas for therapeutic intervention. These areas include the identification of targets for new antifungal drugs and candidates for vaccine development (W. H. Jung, et al., 2006).
4.2 Monothiol Grx4 is required for iron homeostasis and virulence in *C. neoformans*

A preliminary proteomic analysis of potential binding partners for Cir1 was performed in 2009 in our lab, and identification of a monothiol Grx4 (gene ID: CNAG_02950) as a potential Cir1-associated protein set the foundation of the work outlined in my thesis. Monothiol Grxs are reported in other eukaryotic organisms and fungal species to regulate iron homeostasis, iron trafficking and iron signaling events (N. Rouhier, et al., 2010) (H. Li and C. E. Outten, 2012). In the first chapter of my thesis, several lines of evidence indicate that Grx4 in *C. neoformans* has a major role in this process in *C. neoformans*. Importantly, I also demonstrated that in addition to iron homeostasis, Grx4 participates in virulence in *C. neoformans*.

Initially, I demonstrated that Grx4 binds N-terminal region of Cir1 containing the DNA binding domain by ALPHAScreen protein-protein interaction assay and by Y2H analysis. The physical interaction between an iron transcription factor and a monothiol Grx has previously been shown in other fungal species. For instance, interaction between Grx4 and Fep1 in *S. pombe* as analyzed by yeast two hybrid assays has been shown, which is consistent with what I observed in my study (M. Jbel, et al., 2009). They also showed that the conserved C-terminal Grx domain containing the CGFS motif in Grx4 governs iron-dependent modulation of Fep1 activity through close association with the DNA-binding domain. Additionally, in a more recent study by Encinar del Dedo et al., it was ascertained that Grx4 incorporates a glutathione-containing Fe-S cluster and participates in iron starvation signaling by binding and controlling the functions of Fep1 repressor through metal transfer (J. Encinar del Dedo, et al., 2015).
It is important to investigate the cellular compartment for the interaction between Grx4 and Cir1 that was identified in this study. According to observations made by Dr. Guanggan Hu in our laboratory, localization of Cir1 is unaffected by iron status when expressed in *C. neoformans* under both iron-limiting and iron-replete conditions (Dr. Guanggan Hu personal communications). Given the nuclear localization of Cir1 and the high amino acid sequence identity between Cir1 and Fep1, I hypothesize that Grx4 interaction with Cir1 most likely takes place in the nucleus. In this theoretical scenario, Grx4 has an inhibitory influence on Cir1 function by binding Cir1 in presence of iron and dissociating from Cir1 under iron-starvation conditions.

Furthermore, the Fe-S binding capacity of monothiol Grxs via CGFS motif plays a critical role for their functions in iron homeostasis. A report by Muhlenhoff et al. revealed the function of Grx4 homologs Grx3/Grx4 in *S. cerevisiae* in iron trafficking and iron sensing (U. Muhlenhoff, et al., 2010). It was also shown that Grx3/Grx4 form an iron-sensor complex via their bound GSH and Fe-S clusters (Muhlenhoff, 2010) (B. Hoffmann, et al., 2011). It has also been shown that Grx4 is required for iron limitation-dependent inhibition of Fep1 via Fe-S binding capacity of Grx4 in *S. pombe* (M. Jbel, et al., 2009) (J. Encinar del Dedo, et al., 2015). Therefore, it is also possible that Grx4 in *C. neoformans* serves as an iron sensor relaying the levels of iron to Cir1 in the nucleus by shuttling between the cytosol and nucleus. Whether and how Fe–S or Fe is involved in the Grx4-Cir1 interaction is a challenging question to be tackled in the future.

Next, I sought to determine the role of conserved Grx domain of Grx4 in *C. neoformans*. I used a *grx4* disruption strain in which the C-terminal conserved Grx domain was deleted and I generated the complemented *grx4Δ::GRX4* strain with the deleted Grx
domain integrated back in locus. Transcriptome and phenotypic analysis of the grx4 mutant consolidated the role of Grx4 in iron homeostasis in C. neoformans. Remarkably, I found that loss of conserved C-terminal Grx4 domain in GRX4 leads to elaboration of similar iron-related phenotypes seen in cir1 mutants, as described by Jung et al. 2006 (W. H. Jung, et al., 2006).

Regulation of iron homeostasis largely depends on the regulation of iron uptake, iron storage and consumption in response to iron (J. W. Kronstad, 2013) (J. W. Kronstad, et al., 2013) (W. H. Jung and J. W. Kronstad, 2008). In fungi, iron starvation increases expression of genes required for iron acquisition and siderophore-mediated iron uptake, Meanwhile, iron-consuming pathways are downregulated to optimize the use of the limited iron resources (B. Mei et al., 1993) (H. Haas, 2003) (D. L. Greenshields et al., 2007); (M. Schrettl et al., 2007). One key finding from my analysis was based on the gene expression data I obtained for the grx4 mutant compared to WT in presence of iron (Figure 2.11). I found that Grx4 had an inhibitory effect on the expression of iron acquisition gene because these genes were highly upregulated in the grx4 mutant compared to WT in the presence of iron. The regulated functions included iron reductases, siderophores transporters, and components of the high affinity uptake system. Given the aforementioned conserved role of monothiol Grxs in iron homeostasis, it is possible that similar to the scenario in S. pombe, Grx4 in C. neoformans serves as an inhibitory partner for Cir1 in response to iron by physically binding Cir1 and/or Fe-S clusters. This can also result from an influence of Grx4 on downstream regulators that transcriptionally or post-transcriptionally control the genes for iron acquisition in C. neoformans. Therefore, presence of additional levels of iron regulation that have not yet been identified in C. neoformans is possible.
Importantly, evidence from my transcriptional analysis by RNA-seq indicated that Grx4 is required for the repression of many genes that encode iron-dependent proteins, especially those involved in the TCA cycle, respiration, and Fe-S cluster assembly in iron-replete conditions. RNA-seq profiling revealed and confirmed that Grx4 is essential for iron starvation–triggered down regulation of genes in iron-dependent pathways and iron-triggered down regulation of genes for iron acquisition functions. Thus, a key role of Grx4 in iron homeostasis of C. neoformans is to shut down iron-consuming processes, such as respiration, amino acid metabolism, and heme biosynthesis, when iron becomes limiting. Collectively, these results show an impact of Grx4 in repression of iron-dependent pathways and also suggest the essential role of Grx4 in fungal adaptation to iron starvation conditions.

The role of Grx4 in iron homeostasis is further emphasized by my phenotypic analyses. In this context, phleomycin is an antibiotic that damages nucleic acids in the presence of iron (K. R. Fox et al., 1987) (J. Kross et al., 1982). The grx4 mutant exhibited increased sensitivity to the drug presumably as a consequence of high levels of iron uptake activity. I also demonstrated that the grx4 mutant has elevated cell surface reductase activity. This was observed by the enhanced reduction of TTC to 1,3,5-triphenyl-formazan, which appears as a pink-red color colony on media with 0.1% TTC. It has been shown that loss of Cir1 results in elevated cell surface metalloreductase activity, likely as a consequence of lack of transcriptional downregulation of iron acquisition (W. H. Jung, et al., 2006). I also observed that, similar to the cir1 mutant, the grx4 mutant displayed sensitivity to elevated iron levels in plate assays.

I also studied the growth phenotype of the grx4 mutant in the presence of iron in
liquid cultures by growth curves analysis. In this analysis I found that iron-starved grx4 mutants did not respond to iron. This suggested that iron starvation might trigger a non-responsive state in grx4 mutant. Remarkably, I found that supplementation with GSH seemed to restore the capacity of grx4 mutant to perceive iron in high iron medium. This suggests that Grx4 is necessary for efficient growth of C. neoformans in response to iron

Importantly, I demonstrated that loss of the conserved C-terminal Grx4 domain in a grx4 mutant lead to elaboration of the similar virulence-related phenotypes to cir1 mutants shown by Jung et al. 2006 (W. H. Jung, et al., 2006). It was previously shown that cir1 deletion mutants have a defect in capsule formation, altered melanin production, reduced growth at 37°C, and are avirulent in a mouse model (W. H. Jung, et al., 2006). Given the role of iron in regulating the expression of virulence factors in C. neoformans, it is likely that the perturbed iron homeostasis in grx4 mutant is the underlining reason for impaired virulence expression in this mutant, especially because grx4 mutants were unable to colonize and kill mice. This the first study that clearly demonstrates the role of a monothiol Grx as a virulence determinant in a pathogenic fungus. Taken together these results showed the role of Grx4 in both iron homeostasis and virulence n C. neoformans, and suggest that Grx4 may play a key role in transcriptional reprogramming under severe iron limitation encountered in the host. Overall, deregulation of genes required for metabolic adaptation to iron deficiency may account for the impaired ability of grx4 mutant to proliferate in the host. My findings also suggest that Grx4 might be involved in sensing iron levels in the host and expression of iron starvation–induced virulence factors during growth of C. neoformans in the host. Further characterization of influence of Grx4 on virulence targets will provide new insights into the conserved role of the Grx4-
mediated iron homeostasis in cryptococcal pathogenicity in humans.

4.3 GSH is involved in stress response and virulence in *C. neoformans*


Glutathione is an essential metabolite in eukaryotes and is present in high concentration up to 10 mM in yeasts and filamentous fungi (R. C. Fahey and A. R. Sundquist, 1991) (A. Meister and M. E. Anderson, 1983). The classical function of GSH in oxidative stress defense mechanisms includes response to starvation, heat, cold, osmotic shock, and exposure to ROS (D. J. Jamieson, 1998) (V. I. Lushchak, 2012). In this context,
GSH synthetases including Gsh1 and Gsh2, Glr, Gsts, Gpx and Grxs are directly involved in the elimination of oxidative compounds in yeast and other fungi (D. J. Jamieson, 1998) (P. Moradas-Ferreira and V. Costa, 2000). It has been proposed that the glutathione system is critical for resistance to oxidative and nitrosative stress in C. neoformans (T. A. Missall, et al., 2004a). Cooperation between glutathione system and the thioredoxin system in defense against oxidative stress has also been proposed (T. A. Missall, et al., 2004a). However, the role of GSH in cell physiology and metabolism of C. neoformans has not been explored to date.

The role of GSH is not only restricted to cellular redox cycle, but is also required for maintenance of iron homeostasis, mitochondrial structure, and membrane integrity. In fungi GSH is also used as a nutritional supply under sulfur or nitrogen starvation. In this context, it has been shown that GSH is required for adaptation to carbon, sulfur, and nitrogen starvation stress in fungi. These conditions trigger induction of GSH-dependent elements of the antioxidant defence system, leading to an increased tolerance to oxidative stress (M. T. Elskens et al., 1991) (D. Mendoza-Cozatl et al., 2005) (J. Amich et al., 2013, G. A. Marzluf, 1997, I. Pocsi et al., 2004). GSH is also involved in detoxification of harmful intermediates such as methylglyoxal, which is a by-product of glycolysis by the glyoxalase pathway in yeast (A. M. Martins et al., 2001). GSH-dependent detoxification processes are also required for elimination of xenobiotics and antifungal drugs through chemical reaction between GSH and the antifungal drugs (B. Maras, et al., 2014).

One approach to determine the role of GSH is utilization of GSH-depleting drugs, or alternatively deletion of genes in GSH synthesis pathways using genetic methods, followed by analysis of the consequences arising from GSH deficiency. In the study outlined in
chapter 3 of my thesis, GSH deficiency was obtained by deletion of *GSH2* in *C. neoformans*, and the role of GSH biosynthesis in *C. neoformans* was studied.

Phenotypic analysis of *gsh2* mutants grown under non-stressed conditions in YPD and YNB revealed that *GSH2* is dispensable for growth under normal conditions, despite significantly low levels of GSH in these mutants. I speculate this is most likely due to accumulation of product of *GSH1* (dipeptide γ-L-glutamyl-L-cysteine). It is also possible that GSH is not required under non-stressed conditions because Trxs are potentially overproduced to compensate for GSH. Importantly, a functional link between the GSH/Grx and Trx systems in yeast under normal growth conditions has been shown. On this note in normal growth conditions accumulation of ROS in cells is considered low and not detrimental (T. Draculic et al., 2000) (E. W. Trotter and C. M. Grant, 2003) (E. G. Muller, 1996).

Overall, growth of *gsh2* mutants under non-stressed conditions clearly demonstrate that GSH is not essential under these conditions given the very low levels of GSH measured in these mutants. However my studies on *gsh2* mutants that underwent iron starvation led to a deeper understanding of the role of Gsh2 via GSH in *C. neoformans*. I found that iron-starved *gsh2* mutants were auxotrophic for GSH and only supplementation of GSH and not iron, restored growth in these phenotypes. In this context, it had previously been shown that iron starvation leads to ROS production and/or depletion of intracellular GSH pool in *A. thaliana* (L. Ramirez, et al., 2013). Iron is a major constituent of electron transport chain in mitochondria and chloroplasts, and so iron deficiency can lead to disruption of a normal electron transfer resulting in the overproduction of ROS. Consequently, antioxidant systems are required to combat the generated ROS. It was also shown that GSH treatment
counteracted iron deficiency-induced ROS accumulation in *A. thaliana* leaves and led to restoration of ROS to basal levels in presence of GSH (L. Ramirez, et al., 2013). Similarly, my findings suggest that GSH is required to protect against detrimental effects of iron deficiency in *C. neoformans*. I also found that introduction of GSH to iron-starved *gsh2* mutants led to a statistically significant increase in GSH levels in *gsh2* mutants. This suggests that GSH uptake system is present and functional in *gsh2* mutants in *C. neoformans*. In this context, it has been shown that GSH is taken up from the environment by homologues of small oligopeptide transporters (OPT family of proteins) in fungi and plants. Assimilation of radioactively labeled forms of GSH from media led to the discovery of OPT homologues known as high affinity plasma membrane GSH transporter (Hgt1p) in yeast (T. Miyake et al., 1998) (A. Bourbouloux et al., 2000) (S. Sato et al., 1994). Hgt1 is a 799-amino acid polypeptide with a predicted molecular mass of 91.6 kDa with 12–14 transmembrane domains (A. Bourbouloux, et al., 2000) (T. Miyake et al., 2002). Homologues of Hgt1p are apparently restricted to plants and fungi, including *C. albicans* and *S. pombe*. It has been shown that *hgt1* deletion mutants in yeast do not transport GSH, and *hgt1 gsh1* double mutants are not viable (A. Bourbouloux, et al., 2000) (M. A. Lubkowitz et al., 1998, S. Sato, et al., 1994) (M. Hauser et al., 2000). Interestingly, *C. neoformans* genome encodes 5 small oligopeptide transporters with up to 60% of sequence identity to Hgt1 in yeast (encoded by CNAG_019654, CNAG_03013, CNAG_05790, CNAG_04617, CNAG_05737). Therefore analysis of the regulation, functions and contribution of these transporters in the WT and also in *gsh2* mutants background is necessary to fully understand the role of GSH metabolism in virulence inside the host. In regards to virulence, here I demonstrated that *gsh2* mutants were acapsular, non-
melanized and did not grow at 37°C, most likely due to lack of GSH in these mutants. It has previously been shown that elevated temperatures can lead to an increase in the frequency of mutations and interchromosomal DNA recombination, and damages mitochondrial DNA in yeast. Cytotoxic and genotoxic effects of elevated temperature are partially due to oxidative stress and intensified respiration, and it has been shown that GSH is required to counteract these events in yeast, for instance heat shock in S. cerevisiae leads to upregulation of GSH biosynthesis. (J. F. Davidson and R. H. Schiestl, 2001) (K. Sugiyama et al., 2000a) (J. Lee et al., 1997, K. Sugiyama et al., 2000b).

In C. albicans it has been show that glutathione biosynthesis is essential in virulence and disruption of glutathione biosynthesis causes glutathione auxotrophy, elevated (ROS) levels and apoptosis (P. L. Fidel, Jr. et al., 1999) (M. A. Pfaller et al., 2010) (J. R. Wingard, 1995) (Y. U. Baek, et al., 2004) (A. K. Yadav et al., 2011).

Overall in this study, the loss of main virulence traits in gsh2 mutants suggests the presence of a link between glutathione biosynthesis and virulence in C. neoformans. Although the role of GSH transporters is unknown at the moment to predict virulence of gsh2 mutants in animal models or in the host, it is reasonable to propose that C. neoformans exploits the host glutathione for its survival. I predict that despite the presence of the transporter, biosynthesis would still be essential for virulence of C. neoformans in the host depending on the niche that C. neoformans occupies in different stages of its life cycle in the host. For instance, the levels of GSH in the lining fluid of the lungs can reach 300 times the concentration fund in the plasma compared to blood plasma where levels of GSH are exceedingly low (L. J. Smith and J. Anderson, 1992). I speculate that GSH biosynthesis pathway will be most probably required for migration of C.
*C. neoformans* in blood where GSH levels are low. In this case, even the presence of a GSH transporter will not be able to keep up with high glutathione demands of *C. neoformans*, and so the presence of a functional and efficient GSH biosynthesis pathway will become essential.

Overall, given the pleotropic effects of GSH and the numerous cellular processes that can be influenced by GSH deficiency, studying the exact mechanisms that lead to the role of GSH in virulence in the host is important in further understanding of *C. neoformans* pathogenesis.

### 4.4 Conclusion

In conclusion, the studies presented in my thesis demonstrated the critical role of Grx4 in iron homeostasis and virulence in the AIDS-associated fungal pathogen *C. neoformans* var. *grubii*. I also found that glutathione has pleotropic functions with links to iron homeostasis and is required for elaboration of virulence traits in *C. neoformans*. The work presented in my thesis is novel because it is the first study to demonstrate the role of a monothiol Grx in virulence in a pathogenic fungus. Also, it is the first study to describe the role of glutathione in *C. neoformans*. Identifying additional components of iron homeostasis and deciphering their contributions to pathogenesis in *C. neoformans* will serve the ultimate goal of development of new strategies to combat and block cryptococcal infection. This goal could be achieved either by specifically blocking iron-acquisition systems of *C. neoformans* with antifungals, or by reducing the availability of iron in the intracellular niche occupied by *C. neoformans* during the infection.

In the first part of my studies, *in silico* analysis of Grx4 revealed the presence of the conserved C-terminal Grx domain in this protein. The conserved CGFS motif in this domain
is well characterized in non-pathogenic fungal species including *S. pombe* and *S. cerevisiae*. Functional characterization of the *grx4* mutant showed loss of *GRX4* leads to elaboration of iron-related phenotypes including sensitivity to elevated levels and iron-responsive drug phleomycin, also an increased cell surface reductase activity. These phenotypes suggested a perturbed iron homeostasis in absence of Grx4 in *C. neoformans*. Next, differential regulation of iron acquisition genes in the q-RT-PCR and RNA-seq analysis consolidated the role of Grx4 in iron homeostasis and showed that Grx4 is required for transcriptional remodeling of iron acquisition pathways in response to iron. Importantly, loss of *GRX4* led to upregulation of iron acquisition components (including high affinity uptake system, siderophore transporters, and iron reductases) in iron-replete conditions. These results highly suggest that Grx4 has an inhibitory influence on iron acquisition systems in response to iron. I also demonstrated that Grx4 controls the known major virulence factors of the pathogen including the capsule, the formation of the anti-oxidant melanin in the cell wall, and the ability to grow at host body temperature. Remarkably, Grx4 is a major contributor to the disease process, as confirmed by the avirulence of the *grx4* mutant in mice.

### 4.5 Key areas for future directions

#### 4.5.1 A Proposed model of Grx4-Cir1 interaction

The physical binding between Grx4 and Cir1 was clearly demonstrated in ALPHAScreen and Y2H analysis outlined in chapter 2. Given that Cir1 is the master iron regulator in *C. neoformans* (W. H. Jung, et al., 2006), association of Grx4 with Cir1 further highlights the significance of Grx4 in *C. neoformans*. The discovery of association of Grx4 with Cir1 resembles the scenario in *S. pombe* where Grx4 binds Fep1 and is required for
iron-dependent inhibition of Fep1 (M. Jbel, et al., 2011). The exact mechanisms of Grx4-Cir1 binding is not clear at this point but given that grx4 mutant and cir1 mutant share similar iron- and virulence-related phenotypes, and also the fact that Cir1 was initially identified due to high homology to Fep1, it is possible that the interaction between Cir1 and Grx4 in C. neoformans follows similar principles as the mechanism of interaction in Grx4-Fep1 in S. pombe. In addition to the role of Grx4 in Cir1-mediated iron regulation, I also predict a potential role for Grx4 in cellular iron trafficking and iron sensing by incorporating glutathione-containing Fe-S clusters. The conserved property of monothiol Grxs in binding glutathione-containing Fe-S clusters has been fully described in S. pombe and S. cerevisiae (J. Encinar del Dedo, et al., 2015, H. Li and C. E. Outten, 2012).

Previous genetic and biochemical studies have revealed that regulation of iron metabolism in fungi is a multi-step pathway involving proteins in the mitochondria, cytosol, and nucleus (D. H. Howard, 1999). The components of iron homeostasis in C. neoformans have been well characterized and it has previously been shown that Cir1 governs the regulation of iron homeostasis. Here I identified Grx4 as an additional piece of the iron homeostasis puzzle in C. neoformans that most likely exerts its function in iron homoeostasis through interaction with Cir1. I propose a model in which Cir1 is constitutively localized to the nucleus and Grx4 is required to regulate the activity of Cir1 through specific protein–protein interactions. In my proposed model, Grx4 is required to inhibit Cir1 from repressing its target iron acquisition genes under iron-depleted conditions. In the absence of Grx4, Cir1 loses the ability to sense the iron-deplete intracellular environment, which leads to constitutive repression of iron acquisition. In my proposed model iron-dependent modulation of Cir1 activity is exerted through Grx4
binding the N-terminal DNA binding domain composed of a zinc finger and a cysteine-rich region (Figure 4.1).

![Diagram of Grx4-Cir1 interaction](image)

**Figure 4.1: A schematic model of Grx4-Cir1 interaction.** Grx4 binds Cir1 in the nucleus and alters its activity by unknown mechanisms, in response to iron availability. Under low iron replete conditions, Grx4 is required for function of Cir1 in repression of iron acquisition genes. Fe-S or Fe-binding capacity of Grx4, might play a critical role for its interaction with Cir1 and iron homeostasis. Whether and how Fe-S or Fe is involved in this interaction is a challenging question to be tackled in the future.

### 4.5.2 A proposed crosstalk model for GSH functions

In the second part of my studies I elucidated the roles of glutathione synthetase Gsh2
and found that product of this gene, glutathione, remarkably affects a plethora of biological processes and functions with key implications in iron-starvation and virulence in *C. neoformans*. Here, results from analysis of consequences of GSH depletion by deleting *GSH2* suggested that GSH is required for growth of *C. neoformans* in iron-restricted conditions representative of the conditions encountered by this pathogen in the host. I also showed that GSH is required for expression of major virulence traits including capsule formation, melanin production and growth at human body temperature at 37°C. Furthermore, I found that GSH is required for resistance to the major antifungal drugs, cell wall integrity and mitochondrial functions in *C. neoformans*.

*C. neoformans* is subjected to numerous extreme conditions in the host including but not limited to, osmotic and thermal changes, exposure to ROS, nutrient deprivation (including particularly). An efficient and dynamic cross talk among cellular events and pathways that counteract the effects of harsh environment of the host is essential to facilitate the overall cellular integrity of *C. neoformans* and to enhance the capabilities of the *C. neoformans* to proliferate in the host. These pathways include iron homeostasis components and signaling pathways such as the calcineurin, high-osmolality glycerol, and PKC1 pathways, and numerous additional proteins (J. Kronstad, et al., 2012, J. W. Kronstad, et al., 2011). In the second part of my thesis, the findings on the pleotropic role of GSH and the number of cellular events suggested to require GSH, leads to the conclusion that GSH is a fascinating clue to cross talk among these pathways in *C. neoformans*.

Glutathione is the major cellular antioxidant, detoxifier, and an important component both in prokaryotic and eukaryotic cells. Many of the roles of the GSH have been established in fungi as well (I. Pocsi, et al., 2004). In the last decade, the focus of
research on GSH has shifted from its prime role in thiol-redox control, towards its essential requirement in iron homeostasis. Emerging evidence has shown the association of GSH with monothiol Grxs via binding Fe-S clusters and serving as a vital component of iron sensing complexes in yeast and fission yeast (D. T. Mapolelo, et al., 2013) (J. Encinar del Dedo, et al., 2015) It is also established that GSH is essential for delivery and distribution of iron in eukaryotic cells and serves as a major cytosolic ligand for binding ferrous form of iron in the labile iron pool (M. Kruszewski, 2003) (C. C. Philpott and M. S. Ryu, 2014). Also, glutathionylation appears to be a common mode of post-translational modification of iron-dependent transcription factors in both prokaryotes and eukaryotes (C. L. Grek, et al., 2013). Furthermore, GSH depletion has been shown to cause an impairment of extra-mitochondrial Fe-S cluster assembly machinery (ISC) and activation of Aft1 in yeast (K. Sipos, et al., 2002, J. C. Rutherford, et al., 2005). These findings have established GSH as part of the mitochondrial ISC export process and a signal for regulation of iron homoeostasis in yeast (O. S. Chen et al., 2004, U. Muhlenhoff, et al., 2010). Furthermore, intracellular GSH hinders progression of heavy metal-induced cell injuries by scavenging the metal ions themselves and/or by eliminating ROS (P. Perego and S. B. Howell, 1997, S. V. Avery, 2001, C. Cobbett and P. Goldsbrough, 2002).

Based on the findings on the role of GSH in protection against iron-deprivation induced oxidative stress and a plethora of cellular functions in C. neoformans, it is possible to propose a model in which GSH establishes a functional crosstalk between iron homeostasis and cellular function in C. neoformans (Figure 4.2). Given that GSH synthesis and Cir1 influence iron homeostasis and similar cellular functions, interplay between Cir1 and GSH synthesis is suggested in this model. Additionally, according to the mounting evidence on
role of GSH in coordinating Fe-S clusters, it is possible that GSH synthesis links Grx4 and Fe-S cluster coordination (W. Qi et al., 2012). As proposed in Figure 4.1 Grx4 most likely coordinates Fe-S clusters leading to its association with Cir1 through the GRX-like domain. This interaction would induce conformational changes that subsequently modulate Cir1 activity.

Taken together in this model, it is proposed that GSH serves as a core in maintenance of cellular homeostasis in *C. neoformans*. GSH physiological role is split between a function in iron homeostasis by coordinating Fe-S cluster, binding Grx4, and maintaining a vital thiol-redox balances needed for essential cellular functions.
Figure 4.2: Schematics of the proposed functional crosstalk among GSH, iron homeostasis and cellular functions in *C. neoformans*. Grx4 is required for iron homeostasis (blue line). Cir1 has an established role in iron homeostasis and cellular functions (pink line). Grx4 is proposed to form a complex with GSH and/or Cir1 by coordinating Fe-S clusters. The CGFS motif in the conserved C-terminal domain in Grx4, and CXXC motif in the DNA binding region of Cir1 are shown. Similar to Cir1 and Grx4, GSH is essential for key cellular pathways in *C. neoformans*.

Further studies are required to understand the likely partnership between Grx4
and GSH and its role in iron homeostasis. In a broader context, this will contribute to insights into the intricate links between redox imbalance, GSH depletion, alterations of iron homeostasis, oxidative stress and virulence in C. neoformans. This will provide concepts for new, more effective treatments for cryptococcosis, perhaps involving synergistic combinations of oxidant drugs, inhibitors to deplete glutathione or antifungals.

4.6 Future experiments

4.6.1 Study the interaction of Grx4 with Cir1 in C. neoformans

Chapter 2 of this thesis demonstrated that Grx4 binds Cir1 in ALPHAScreen and Y2H assays. However the molecular events of this interaction in C. neoformans and whether iron levels have a role in this interaction was not determined. This could be investigated by in vivo co-immunoprecipitation. This requires construction of strain that harbour tagged Cir1 and Grx4. For instance, CIR1::GFP fusion allele is already available and has already been constructed by Dr. Guanggan Hu. Generation of mCherry-Grx4 fusion for instance can be a good place to start. This will also involve preparation of lysates from the strains grown in low-iron medium or high-iron medium followed by use of anti-GFP and anti-dsRed antibodies in Western blot analysis. Alternatively, the generated strain can be used to investigate the Cir1-Grx4 interaction by confocal microscopy. Additionally, these strains could be used to determine the localisation of Grx4 in iron-deplete or iron-replete conditions by subcellular fractionation followed by Western blot.

4.6.2 Investigating Grx4 capacity to assemble Fe-S clusters

As discussed in chapter 2, the function of monothiol Grxs with conserved CGFS motif in iron regulation has been extensively studied in S. cerevisiae and S. pombe. According to recent line of evidence, recombinant versions of bacterial, human, yeast and plant
monothiol Grxs orthologues produced in *E. coli* are able to incorporate Fe–S clusters. This complex has a labile nature and detection of Fe–S clusters bound to these CGFS Grxs require specific analytical and biochemical techniques, such as spectroscopic UV-visible absorption/circular dichroism (CD), resonance Raman and Mössbauer analyses of anaerobically purified proteins or proteins repurified after in vitro Fe–S cluster reconstitution of apo-proteins. It will be important to investigate whether Grx4 assembles Fe–S cluster. If it is proven that Grx4 can undergo transition between a holo- and apo-Grx4, then this will ascertain a suggested role of Grx4 as the regulatory switch in the complex iron regulatory network of *C. neoformans*. Investigation of Fe–S cluster reconstitution on Grx4 will require advanced biochemical, spectroscopic and analytical techniques to examine binding of recombinant Grx4 to Fe–S clusters. This will also involve purification of recombinant Grx4 under anaerobic conditions to minimize loss of the Fe–S cluster. Also, reconstitution of Fe–S clusters on apo-Grx4 needs to be carried out under anaerobic conditions (O$_2$ < 5 ppm) according to previously published protocols (R. Lill and U. Muhlenhoff, 2006) (H. Li, et al., 2009) (U. Muhlenhoff, et al., 2010, B. Zhang, et al., 2013) (P. Shakamuri et al., 2012).

### 4.6.3 Further understanding of the transcript structure of the *GRX4* locus

Re-analysis of data obtained from RNA-sequencing outlined in chapter 2 indicated the presence of several transcript versions (with variation in N-terminal region) arising from the *GRX4* locus. The identification of several transcripts from *GRX4* locus in accumulating RNA-Seq data raised the possibility that there is more than one transcript from the *GRX4* locus. Future work is required to thoroughly investigate the structure of the *GRX4* locus. This analysis involves preparing cDNA from WT and *grx4* mutant cells grown
under different conditions, using carefully designed primers at the boundaries of each predicted intron and exon to amplify sequences from cDNA and genomic DNA. This can be further evaluated either by Northern analysis using specific primer radiolabeled with [α-\textsuperscript{32}P] dCTP, or by Western blot analysis with anti-Grx4 antibody and extracts from wild type and the grx4 mutant to understand the protein(s) expressed from the locus. Given that numerous efforts to delete the N-terminal region in \textit{C. neoformans} reference strain H99 (haploid background) by biolistic transformation were unsuccessful, and taking into account that N-terminal region of the \textit{GRX4} locus is not essential in a stable diploid background, it would be interesting to determine whether there is a highly intricate regulation of gene expression in the GRX4 locus in particular in the N-terminal variable region, in \textit{C. neoformans} It would also be interesting to find out whether these versions with variation in N-terminal region contribute to different functions under low iron or high iron condition or in different cellular compartments. Future work is required to determine whether transcript variability results from alternative splicing or alternative transcription start sites and if alternate isoforms are biologically relevant.

\subsection{Investigation of reversible S-glutathionylation of Cir1}

An increasing body of information supports the idea that monothiol Grxs regulate the activity of their targets by reversible glutathionylation. As discussed in chapter 2 of this thesis, one of my speculations on the mechanism Grx4-Cir1 interaction was related to the possibility of Cir1 being post-translationally modulated by Grx4 de-glutathionylating activity. Future work is required to investigate the regulation of Cir1 by reversible S-glutathionylation. This will constitute an emerging and novel area of research; especially that since reversible S-glutathionylation in \textit{C. neoformans} has never been reported.
Investigation of this phenomenon in regulation of Cir1 due to the established role of Cir1 as the Achilles heel of *C. neoformans* in regulation of iron acquisition and cellular functions including virulence factor expression adds to the value of this future experiment.

### 4.6.5 Construction and phenotypic characterization of GSH transporter deletion mutants

Glutathione has key roles in a plethora of essential functions in fungi (I. Pocsi, et al., 2004). In general My findings in chapter 3 provide considerable insight and additional knowledge about glutathione’s cellular and metabolic functions in *C. neoformans*. However, they are just the tip of the iceberg; future glutathione research will lead to novel discoveries in cryptococcal biology and pathogenesis.

One of my key findings was that *gsh2* mutants were acapsular, non-melanized and did not grow at 37°C, and I speculated that this is most likely due to lack of GSH in these mutants. Future work is required to investigate the virulence capacity of *gsh2* mutant in animal models. At present, the role of GSH transporters are unknown and therefore it is important to determine the role of these transporters in *C. neoformans* by generating deletions of their corresponding genes in the genome. Therefore analysis of the regulation, functions and contribution of these transporters in the WT and also in *gsh2* mutants background is necessary to fully understand the role of GSH metabolism in virulence inside the host.

### 4.6.6 Investigation of Grx4 and GSH crosstalk in *C. neoformans*

Collectively, my findings showed that both Grx4 and GSH have critical roles in iron homeostasis in *C. neoformans*. In chapter 4, I suggested possible crosstalk between Grx4 and GSH that influence functions in iron homeostasis and virulence in *C. neoformans*. It is
highly likely that some of the roles of Grx4 are mediated through an GSH dependent mechanism, and alternatively Grx4 might form a stable complex with GSH as shown in other *S. pombe* and *S. cerevisiae*. To test these possibilities and determine potential redundant roles of Grx4 and Gsh2 in iron homeostasis will require generation of double *grx4gsh2* mutants.
References


Appendices

Appendix A

Table A1: List of proteins identified in Cir1 binding assay identified by Mass-Spectrometry in an experiment performed by Dr. Horacio Bach.

<table>
<thead>
<tr>
<th>Broad ID</th>
<th>Protein</th>
<th>Domains found/ Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNAG_04158</td>
<td>Transcription co-repressor</td>
<td>-Tup1-like (enhances split, repression of chromatin by repression of histones genes)</td>
</tr>
</tbody>
</table>
| CNAG_03018 | Nuclear protein (transcription regulation) | - Fungal Zn(2)-Cys (6) binuclear cluster  
- Fungal specific transcription factor domain |
| CNAG_02426 | Spliceosome complex protein      | - Tetraicopeptide repeat  
- HAT (half-A-TRP) important for complexes in RNA processing |
| CNAG_03761 | mRNA3’end-processing RNA 14     | - PT repeat  
- Tetraicopeptide repeat (mediates protein-protein interactions)  
- Suppressor of forked protein (SUF) |
| CNAG_03987 | eRFS                            | - Elongation factor Tu GTP binding domain  
- Elongation factor Tu domain 2  
- Elongation factor Tu C’ terminal domain |
| CNAG_00708 | Pre-mRNA-splicing factor SLT11   | - Zinc finger C-X8-C-X5-C-X3 H type                                                      |
| CNAG_06773 | Protein Transport SEC 24        | - Sec23/Sec24 Zinc Finger (COPII coated vesicles ER=>Golgi) Binds Sar1, Sec23/24, Sec13/31 in complex  
- Gelsolin repeat (binds to barbed ends of actin filaments and fibronectin) |
| CNAG_02178 | Cog3                             | - Sec 34-like family                                                                    |
| CNAG_03582 | pH-response regulator palA/RIM20 | - BRO1-like domain (endosomal targeting; ESCRT III subunit Snf7 binds to BRO1 for protein sorting function (VPS32)) |
| CNAG_03372 | VPS-BRO1                         | - BRO-like protein (in sorting multivesicular body)                                      |
| CNAG_04943 | CBS domain-containing protein    | - DUF 21  
- CBS domain pair (forms stable globular domains, binds adenosyl groups) |
<table>
<thead>
<tr>
<th>Broad ID</th>
<th>Protein</th>
<th>Domains found/ Comments</th>
</tr>
</thead>
</table>
| CNAG_04303 | E3 ubiquitin-protein ligase BRE1 | - DUF 1610  
                  - Zn finger C3HC4 type (Ring finger, in ubiquitation pathway)  
                  - BRE E3 ubiquitin ligase transcriptional activator |
| CNAG_05005 | ULK/ULK Protein kinase | - Tyrosine kinase  
                  - MIT (microtubule interacting and transport, binds ESCRT III endosomal sorting complexes) |
| CNAG_01655 | Dynamin | membrane transport |
| CNAG_03319 | Phospholipid binding protein | - SH3 domain  
                  - SAM domain (sterile alpha motif, in protein-protein interactions)  
                  - PH domain (pleckstrin homology, in intracellular signaling or cytoskeleton) |
| CNAG_01185 | Hsp70-like protein | - HEAT repeat  
                  - Nucleotide exchange factor FES1 |
| **CNAG_02950** | **Grx4** | **- Glutaredoxin domain** |
| CNAG_05730 | Acyl protein thioesterase 1 | - Phospholipase |
| CNAG_03853 | Small COPII coat GTPase SAR1 (see 6773) | - ADP ribosylation factor family  
                  - MIRO-like protein |
| CNAG_01054 | Phosphoprotein phosphatase | - SSU72 domain (binds TFIIB, involved in polyadenylation and termination) |
Figure A.1 Functional categorization of Cir-1 potential binding partners

- **Others**
  - CNAG_01655- Dynamin
  - CNAG_03319- Phospholipid-binding protein
  - CNAG_01185- Hsp70-like
  - **CNAG_02950- Monothiol Grx4 Family**
  - CNAG_05730- Thioesterase
  - CNAG_03853- Sar1
  - CNAG_01054- Phosphoprotein phosphatase

- **Endocytosis**
  - CNAG_03582- pH-response regulator
  - CNAG_03372- VPS-BRO1
  - CNAG_05005- Tyrosine kinase
  - CNAG_04943- CBS
  - CNAG_04303- BRE1
  - CNAG_01583- Sarf7

- **Transport**
  - CNAG_06773- Sec24
  - CNAG_02178- Sec34

- **Transcription & RNA processing**
  - 4158- Tup-1 like
  - 3018- Transcription regulator
  - 2426- Spliceosomal complex
  - 3987- Elongation factor
  - 0708- Pre-mRNA- splicing factor

**Cir1**
Table A2: Categories of differentially expressed genes based on GO terms associated with molecular function in the *grx4* mutant compared to WT cells grown under the iron-deplete condition.

<table>
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<tr>
<th>Go terms*</th>
<th>H99 Gene ID</th>
<th>Gene annotation</th>
<th>Fold change (Low-iron)*&lt;sub&gt;grx4Δ vs WT&lt;/sub&gt;</th>
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<td>oxidoreductase activity (GO:0016491)</td>
<td>CNAG_01323</td>
<td>ubiquinol-cytochrome-c reductase</td>
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<td>CNAG_01991</td>
<td>cytochrome c oxidase subunit V</td>
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<td>CNAG_00462</td>
<td>oxidoreductase</td>
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<td>CNAG_04417</td>
<td>2,4-dichlorophenoxyacetate alpha-ketoglutarate dioxygenase</td>
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<td>CNAG_04471</td>
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<td>CNAG_01229</td>
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<td>CNAG_03465</td>
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<td>CNAG_05169</td>
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<td>CNAG_02266</td>
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| Go terms* | H99 Gene ID | Gene annotation | Fold change (Low-iron)*
|-----------|-------------|-----------------|-----------------
|           |             | CNAG_06976      | grxAΔ vs WT   |
|           |             | ferric-chelate reductase | -3.51     |
|           |             | CNAG_02592      | thioredoxin reductase GliT | -7.83     |
| Fe-S cluster binding (GO:0051536) | CNAG_07177 | NADH dehydrogenase Fe-S protein 3 | 3.48     |
|           |             | CNAG_01137      | aconitase     | 3.67     |
|           |             | CNAG_04358      | Fe-S clusters transporter ATM1 | 3.78     |
|           |             | CNAG_02315      | ubiquinol-cytochrome c reductase Fe-S subunit | 3.84     |
|           |             | CNAG_07908      | aconitate hydratase | 6.59     |
|           |             | CNAG_04202      | iron hydrogenase | 15.51    |
|           |             | CNAG_03226      | succinate dehydrogenase Fe-S subunit | 27.12    |
| Metal cluster binding (GO:0051540) | CNAG_01133 | mitochondrial protein | 3.00     |
|           |             | CNAG_05179      | ubiquinol-cytochrome c reductase complex core protein 2 | 3.38     |
|           |             | CNAG_01915      | ribonucleotide reductase subunit | 3.52     |
|           |             | CNAG_00022      | holocytochrome-c synthase | 4.22     |
|           |             | CNAG_05909      | electron transporter | 5.83     |
|           |             | CNAG_07862      | fumarate reductase | 8.49     |
|           |             | CNAG_00716      | electron carrier | 18.22    |
|           |             | CNAG_01464      | flavohemoglobin | 26.45    |
|           |             | CNAG_05875      | cytochrome c heme lyase | -4.33    |
Table A3: Categories of differentially expressed genes based on GO terms associated with molecular function in the *grx4* mutant compared to WT cells grown under iron-replete condition.

<table>
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<tr>
<th>Go terms*</th>
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<th>Fold change (High-iron)*</th>
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<td>glyceraldehyde 3-phosphate dehydrogenase</td>
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Table A4: Categories of differentially expressed genes based on GO terms associated with molecular function with similar patterns of expression in the *grx4* and *cir1* mutant mutant compared to WT cells grown under iron-deplete condition.

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Table A5: Categories of differentially expressed genes based on GO terms associated with molecular function with similar patterns of expression in the *grx4* and *cir1* mutant mutant compared to WT cells grown under iron-replete condition.

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Table A6: Categories of differentially expressed genes based on GO terms associated with molecular function with distinct patterns of expression in the *grx4* or *cir1* mutants compared to WT cells grown under iron-deplete condition.

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Table A7: Categories of differentially expressed genes based on GO terms associated with molecular function with distinct patterns of expression in the *grx4* or *cir1* mutants compared to WT cells grown under iron-replete condition.

<table>
<thead>
<tr>
<th>Molecular function</th>
<th>H99 Gene ID</th>
<th>Gene annotation</th>
<th>Fold change (High-iron)$^b$</th>
<th>$grx4\Delta$ vs WT</th>
<th>$cir1\Delta$ vs WT</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DNA repair</strong></td>
<td>CNAG_00178</td>
<td>MUS42</td>
<td>5.36</td>
<td>no change</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CNAG_00299</td>
<td>DNA repair protein RAD5</td>
<td>4.43</td>
<td>-2.73</td>
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<tr>
<td></td>
<td>CNAG_00720</td>
<td>recombinase</td>
<td>4.98</td>
<td>-2.59</td>
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<tr>
<td></td>
<td>CNAG_01144</td>
<td>damaged DNA binding protein</td>
<td>3.64</td>
<td>-2.19</td>
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<tr>
<td></td>
<td>CNAG_01163</td>
<td>DNA supercoiling</td>
<td>5.01</td>
<td>no change</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CNAG_02561</td>
<td>spermine transporter</td>
<td>4.59</td>
<td>no change</td>
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<tr>
<td></td>
<td>CNAG_02771</td>
<td>DNA supercoiling</td>
<td>7.16</td>
<td>-3.95</td>
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<tr>
<td></td>
<td>CNAG_02771</td>
<td>DNA supercoiling</td>
<td>3.62</td>
<td>no change</td>
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</tr>
<tr>
<td></td>
<td>CNAG_03923</td>
<td>endonuclease</td>
<td>3.92</td>
<td>no change</td>
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<tr>
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<td>CNAG_04220</td>
<td>Ku70 protein</td>
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</tr>
<tr>
<td></td>
<td>CNAG_06589</td>
<td>endoribonuclease L-PSP</td>
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<td>no change</td>
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<tr>
<td></td>
<td>CNAG_06724</td>
<td>DNA strand annealing</td>
<td>8.48</td>
<td>-2.31</td>
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<tr>
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<td>CNAG_07552</td>
<td>DNA repair protein rad8</td>
<td>8.78</td>
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<td>CNAG_07766</td>
<td>DNA polymerase lambda</td>
<td>7.38</td>
<td>-2.06</td>
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<tr>
<td><strong>Others</strong></td>
<td>CNAG_00036</td>
<td>Sec14 cytosolic factor</td>
<td>-4.20</td>
<td>no change</td>
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<tr>
<td></td>
<td>CNAG_04556</td>
<td>endoplasmic reticulum receptor</td>
<td>3.01</td>
<td>-3.00</td>
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</tr>
</tbody>
</table>
Appendix B

Figure B.1: Deletion of GSH2 in gDNA extracted from 3 individual gsh2Δ mutants and re-integration of GSH2 in locus in gDNA extracted from gsh2Δ::GSH2 in C. neoformans H99 background was confirmed by PCR. Primers amplified a 650 bp region inside GSH2 gene in C. neoformans. This region was amplified from gDNA extracted from the WT strain and and the complemented gsh2Δ::GSH2 strain, no band was amplified when gDNA was extracted from 3 individual gsh2Δ mutants.