

2016

Investigating Cross Talk Between The High And Low Iron Sensors In Saccharomyces Cerevisiae

John S. Hepburn
University of South Carolina

Follow this and additional works at: <https://scholarcommons.sc.edu/etd>

 Part of the [Chemistry Commons](#)

Recommended Citation

Hepburn, J. S. (2016). *Investigating Cross Talk Between The High And Low Iron Sensors In Saccharomyces Cerevisiae*. (Master's thesis). Retrieved from <https://scholarcommons.sc.edu/etd/3850>

This Open Access Thesis is brought to you by Scholar Commons. It has been accepted for inclusion in Theses and Dissertations by an authorized administrator of Scholar Commons. For more information, please contact dillarda@mailbox.sc.edu.

INVESTIGATING CROSS TALK BETWEEN THE HIGH AND LOW IRON SENSORS IN
SACCHAROMYCES CEREVISIAE

by

John S Hepburn

Bachelor of Science
South Carolina State University, 2011

Submitted in Partial Fulfillment of the Requirements

For the Degree of Master of Science in

Chemistry

College of Arts and Sciences

University of South Carolina

2016

Accepted by:

Caryn Outten, Director of Thesis

Thomas Makris, Reader

Paul Allen Miller, Vice Provost and Interim Dean of Graduate Studies

© Copyright by John S Hepburn, 2016
All Rights Reserved.

ACKNOWLEDGEMENTS

First and foremost I would like to thank my advisor Dr. Caryn Outten. She has always been extremely supportive and encouraging in all my endeavors and she continues to be a wealth of knowledge. I am extremely grateful to all the Outten lab members past and present who have mentored and shared science with me as well. We are after all the company we keep. I would also like to thank Dr. Thomas Makris for all of his insights into research and science as well.

ABSTRACT

Iron is essential for nearly all life on earth. Cells must maintain sufficient levels of this important nutrient due its function as a central co-factor for many cellular processes. Although iron is extremely plentiful, its poor solubility requires specialized import. On the other side, too much iron inside the cell can lead to the generation of reactive oxygen species (ROS), which have deleterious effects on many cellular functions and have been implicated in a wide variety of diseases such as cancer and Alzheimer's disease. Using the model eukaryote *Saccharomyces cerevisiae*, or budding yeast, our research group has accomplished much in determining the mechanism of regulation for the low iron-sensing transcription factor, Activator of Ferrous Transport (Aft1), and its paralog Aft2. However, much remains unknown about the mechanism of regulation for the high iron-sensing transcription factor, yeast-activating protein 5 (Yap5). Recent studies in our lab have shown that Yap5, much like Aft1/2, senses iron availability through iron sulfur (Fe-S) cluster binding. Utilizing analytical gel filtration, UV-visible, and CD spectroscopy we have shown that Yap5 and Aft2 interact in vitro and that this interaction is bridged by an iron sulfur cluster transfer. Moreover the transfer has directionality with cluster going from Aft2 to Yap5. This finding is not without physiological repercussions, providing first time evidence that the signal from low to high iron is shared between the two transcriptional regulators and they directly interact with an iron sulfur cluster being a switch in the regulatory response.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS.....	iii
ABSTRACT	iv
LIST OF TABLES	vi
LIST OF FIGURES	vii
LIST OF SYMBOLS	viii
LIST OF ABBREVIATIONS.....	ix
CHAPTER 1 INTRODUCTION.....	1
1.1 SIGNIFICANCE OF IRON.....	1
1.2 IRON HOMEOSTASIS IN S. CEREVISIAE	1
1.3 YEAST ACTIVATING PROTEIN (YAP) FAMILY AND YAP5	4
CHAPTER 2 YAP5 AND AFT2 INTERACTION STUDIES IN VITRO	6
2.1 ABSTARCT.....	6
2.2 INTRODUCTION	6
2.3 EXPERIMENTAL PROCEDURES	8
2.4 RESULTS	13
2.5 DISSCUSSION.....	23
CHAPTER 3 SUPPLEMENTARY MATERIALS AND METHODS.....	26
REFERENCES	28

LIST OF TABLES

Table 2.1 Primers used for cloning and mutagenesis	9
Table 3.1 Primers used for cloning and colony screening	27

LIST OF FIGURES

Figure 1.1 Model for iron regulation in <i>S. cerevisiae</i>	3
Figure 1.2 Schematic diagram of Yap family proteins	5
Figure 2.1 SDS-PAGE expression analysis of constructs used in this study.....	15
Figure 2.2 Domain structure of Yap5 constructs used in this study	16
Figure 2.3 UV-visible absorption and CD spectroscopy of Holo Yap5	17
Figure 2.4 Analytical gel filtration chromatography following interaction of Aft2 and Yap5	19
Figure 2.5 CD spectra comparison of [2Fe-2S] Aft2 and Yap5	20
Figure 2.6 CD spectra comparison of [2Fe-2S] Yap5 with apo-Aft2 (A) and (B) [2Fe-2S] Yap5 with apo-Grx3.	22
Figure 2.7 Model for Aft2 and Yap5 interaction	25

LIST OF SYMBOLS

- Δ Deletion.
- β IUPC abbreviation for position of carbon atom

LIST OF ABBREVIATIONS

AFT1	Activator of Ferrous Transport 1
AFT2	Activator of Ferrous Transport 2
BOLA	Protein Family Name
BPS	4,7-Diphenyl-1,10-Phenanthrolinedisulfonic Acid
BSA	Bovine Serum Albumin
CCC1	Cross Compliments Ca ²⁺ phenotype
CD	Circular Dichroism Spectroscopy
CRD	Cysteine Rich Domain
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
FRA1	Iron Repressor of Activation 1
FRA2	Iron Repressor of Activation 2
GRX3	Glutaredoxin 3
GRX4	Glutaredoxin 4
IPTG	Isopropyl β -thiogalactoside
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
ROS	Reactive Oxygen Species
SD	Synthetic Defined

SDS-PAGE Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
YAP5 Yeast Activating Protein 5
TCEP..... Tris[2-carboxyethyl] phosphine

CHAPTER 1

INTRODUCTION

1.1 Significance of Iron

Iron is an essential element for nearly all organisms on earth. Iron contributes to numerous cellular processes, including enzymatic reactions, DNA replication and repair, energy metabolism, cellular detoxification, and many others. Iron has the ability to act as both an electron donor and acceptor by switching between the ferrous (Fe^{2+}) and ferric (Fe^{3+}) redox states (6). However, iron is a double-edged sword: although it is necessary for nearly all life on earth, the same properties that make it suitable for redox chemistry also make it possible to participate in the Fenton reaction, generating the highly reactive hydroxyl radical (OH^{\bullet}). (7) Formation of ROS *in vivo* can lead to protein, DNA, and lipid damage. Excessive levels of iron have also been associated with many diseases such as hemochromatosis and neurodegenerative disorders (8). Nevertheless, when iron levels are too low, the cell may not meet all of its biological requirements leading to the impairment of the electron transport chain, oxygen sensing, and DNA synthesis (9). This situation can lead to a host of physiological problems such as anemia, respiratory impairment, and perinatal mortality (10,11). Organisms have thus evolved a myriad of ways to control iron homeostasis in order to maintain critical cellular levels of iron.

1.2 Iron homeostasis in *S. cerevisiae*

Our lab currently uses the budding yeast *Saccharomyces cerevisiae* as model eukaryotic system. *S. cerevisiae* is particularly well suited for numerous reasons.

In short, its smaller genome size facilitates faster results and many of the pathways for iron homeostasis in plants and animals are conserved in yeast (12). Budding yeast can survive under a variety of iron availability environments, as well as being able to grow under both aerobic and anaerobic conditions, which makes this organism ideally suited for use in our studies. *S. cerevisiae* regulates iron homeostasis at the transcriptional, post-transcriptional, and post-translational levels (13). This thesis focuses mainly on transcriptional regulation in response to varying iron levels, specifically the roles of the transcriptional regulators Yap5 and Aft1/2. Under iron-replete conditions, expression of the gene products responsible for various iron transporters is activated by Aft1 and its paralog Aft2, resulting in iron import (4). When iron levels reach a sufficient level, Aft1/2 receive an inhibitory [2Fe-2S] cluster from the monothiol glutaredoxins Grx3 and Grx4 and the BolA-like protein Fe repressor of activation-2 (Fra2) (14, 15, 2). This leads to dimerization and sequestration of Aft1/2 in the cytosol, preventing activation of iron uptake genes and thus protecting the cell from over-accumulation of iron Figure 1.1 (4). On the other end of the spectrum, when iron levels become toxic, Yap5 becomes active and initiates a much smaller set of gene products, including Ccc1 the major vacuolar importer of iron, which in turn provides a means of iron sequestration (16, 17).

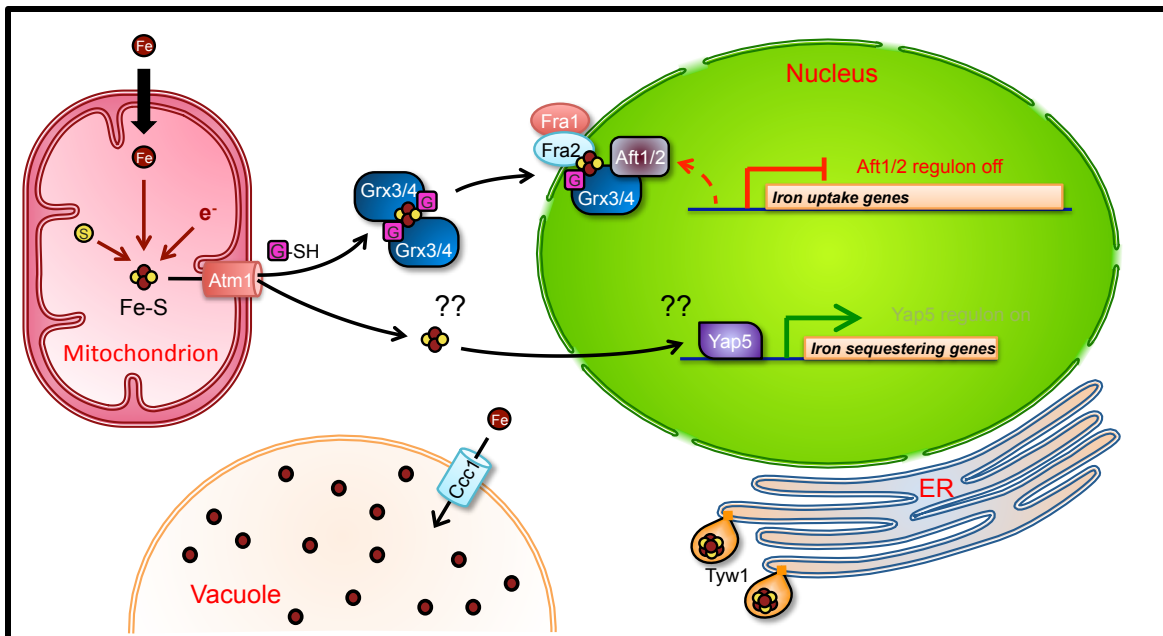


Figure 1.1 Model for iron regulation in *S. cerevisiae*. Under iron replete conditions Aft1/2 are bound to DNA and actively transcribe iron uptake genes. When iron levels are high, Aft1/2 are shuttled out of the nucleus and Yap5 becomes active (4).

1.3 Yeast Activator Protein (YAP) Family and Yap5

Although a great deal of work has gone into understanding the roles that Aft1 and Aft2 play in remodeling gene expression (2, 10, 12), much less is known about Yap5. Yap5 is one of eight proteins in the Yap family of yeast transcriptional activators (1). Proteins in the Yap family alter gene expression in response to varying types of stress ranging from oxidative stress to detoxification responses (1). Proteins in the Yap family contain a basic leucine zipper domain (bZIP) at the N-terminus, which enables them to bind DNA, and a C-terminal activation domain (Figure 1.2) (20). Previous work by the Kaplan group has shown that Yap5 is constitutively bound to its promoter with activation dependent upon an iron signal derived from the production of mitochondrial Fe-S clusters (13,14). It was further shown that when several conserved cysteines near the N-terminus of the activation domain were mutated to alanine, Yap5 was no longer able to regulate its gene targets (14). Since ligation of Fe-S clusters through cysteine residues is common, this led researchers to further probe whether an Fe-S cluster was bound to Yap5. Concurrent with our preliminary data, researchers at the University of Marburg have recently shown that Yap5 does harbor a [2Fe-2S] cluster (20). However, the way in which this Fe-S cluster signal is transferred to Yap5 remains unknown. Interestingly, a large-scale yeast two-hybrid screen has identified a physical interaction between the high and the low iron regulators Aft1 and Yap5 (19). This finding is important, but needed to be demonstrated with *in vitro*. Here we have successfully expressed and purified Aft2 and Yap5 and probed their interaction by analytical gel filtration, UV-visible spectroscopy and CD spectroscopy and have shown that the two proteins interact specifically through

iron sulfur cluster transfer. This finding proves valuable in elucidating the role that iron sulfur clusters play in iron homeostasis.

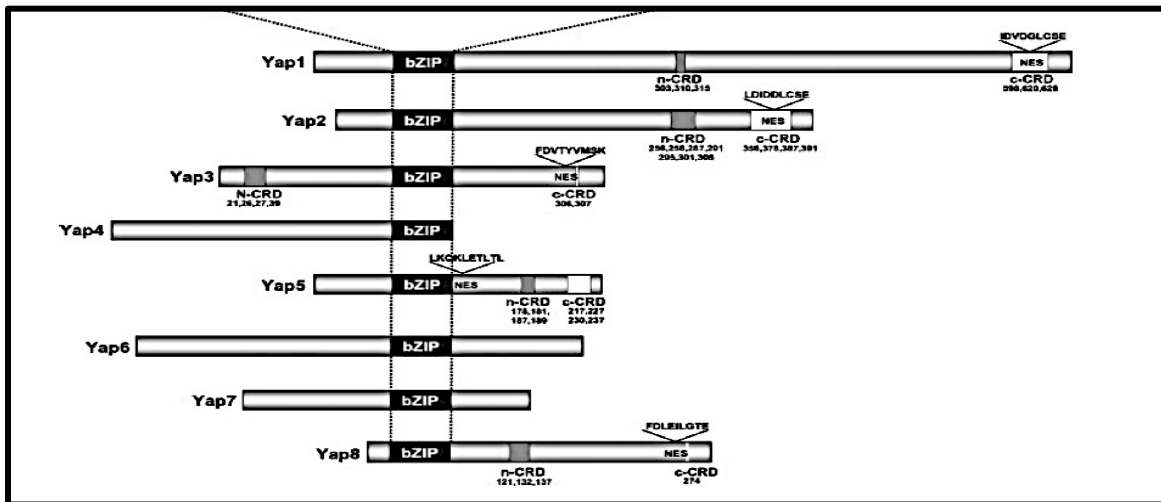


Figure 1.2 Schematic diagram of Yap family proteins. Included in schematic are regions shared between the members, specifically the bZIP domains and the cysteine rich activation domains (CRDs) (13).

CHAPTER 2

YAP5 AND AFT2 INTERACTION STUDIES IN VITRO

2.1 Abstract

Iron homeostasis in yeast is regulated at the transcriptional level by three main transcriptional activators Aft1, Aft2, and Yap5. When iron is deficient in the cell Aft1 and its paralog Aft2 bind to their promoters and activate the transcription of several proteins involved in iron acquisition (2). Aft1/2 have been shown to sense adequate iron availability, through an inhibitory iron sulfur (Fe-S) cluster delivered by a complex of proteins Grx3/Fra1 and Fra2 which in turn leads to dimerization and subsequent shuttling out of the nucleus (12). This is in direct contrast to conditions of iron overload whereby Yap5 senses high levels of iron and in turn activates the transcription of genes responsible for iron storage and utilization (13,14). Recent advances in our laboratory and others have shown that the activation response is due to cluster binding yet the proteins that deliver the activation cluster remain unknown. Our lab has shown with analytical gel filtration, CD, and UV-visible absorption studies that Aft2 interacts with Yap5 *in vitro* and that this interaction is likely through a cluster transfer.

2.2 Introduction

Iron as a cofactor is essential for a wide variety of biological processes ranging from electron transport to oxygen binding to name a few. Iron is one of the most abundant metals on earth, but with the rise of atmospheric oxygen some 2,500 million

years ago its solubility changed from that of ferrous iron to ferric iron causing the majority of iron found on earth to become insoluble (24). The presence of oxygen was not only a problem with the precipitation of iron causing a decrease in bioavailability, but also production of reactive oxygen species. Rather than evolving special pathways to control oxygen saturation, it seems that early life forms set about evolving mechanisms and pathways to control the amount of iron inside the cell and even new and exciting chemistries with iron and various other metals (24). The challenge in understanding these varied pathways of iron homeostasis is vast. Organisms have multiple routes in which they can maintain iron levels and so we must first piece together each one separately and then like a patchwork quilt, sew them together in order to tell a complete story.

Current advances in iron homeostasis have been made more obtainable using *S. cerevisiae* as a model for eukaryotic iron homeostasis. In yeast, the pathway that is widely studied is known as the iron regulon, which is comprised of several proteins, namely Aft1 and its paralog Aft2 at the center. In this pathway when iron levels inside the cell are diminished, Aft1/2 bind to the promoter of a suite of genes involved with iron uptake and activate transcription causing the downstream affect of iron uptake (2). Recent studies conducted in our research group and elsewhere have shown that when iron levels become adequate, a complex of proteins Grx3/4, Fra1, and Fra2 deliver an inhibitory Fe-S cluster to Aft1/2 which leads to its dimerization and subsequent shuttling outside the nucleus (2, 12, 21).

On the other end of iron homeostasis, when iron levels are high enough to become toxic, the cell responds by activating the transcription of several genes involved in iron utilization and storage, which is accomplished via a transcriptional activator Yap5 (2,

12). For many years it was not known what form of iron Yap5 responded to; however, recent studies have shown along with our current research that the response to iron levels is due to an Fe-S cluster (18). Using UV-visible absorption and CD spectroscopy, acid labile sulfur assays, and iron assays we have successfully expressed and purified a truncated version of the Yap5 protein which harbors the activation site and contains a [2Fe-2S] cluster. More interestingly, protein-protein interaction studies using analytical gel filtration and CD spectroscopy demonstrate that Yap5 interacts with Aft2 in vitro. From our results, it is plausible that Aft2 binds a [2Fe-2S]²⁺ cluster and delivers it to Yap5 based on increasing iron levels in the cell. This finding would suggest that the same molecule that leads to inhibition in one case would lead to activation in the other and the two proteins share a direct cross talk with one another culminating in the balance of intracellular iron.

2.3 Experimental Procedures

Plasmid Construction. Construction of the yeast Yap5 open reading frame was accomplished previously in our lab by Chris Bird. Briefly the ORF of the yeast Yap5 gene was amplified from *S. cerevisiae* genomic DNA by PCR with primers included in Table 2.1 and cloned into the NcoI and EcoRI sites of pRSF-Duet1 (Novagen) to generate pRSF-Duet1-Yap5. The plasmid pRSF-Duet1-Yap5Δ1-116 was created by site directed mutagenesis. Briefly, a second NcoI site, which included a start site at position 117, was introduced in pRSF-Duet1-Yap5 using the primers listed in Table 2.1. This plasmid was then digested with NcoI and religated to remove codons for amino acids 1-116. The truncated pRSF-Duet1-Yap5Δ1-161 was created by a similar approach. An second NcoI site, which included a start site at position 162, was introduced in pRSF-

Duet1-Yap5 and the mutated plasmid digested with NcoI. Re-ligation of the plasmid then allows removal of the coding sequence for the amino acids 1-161. This was accomplished using the primers listed in Table 2.1. All plasmids were verified by restriction digest and double-stranded DNA sequencing (Selah Genomics Inc., USC Environmental Core Facility). The plasmid pET30a-Aft2(1-204) was kindly previously supplied to our lab by Dr. Chuan He from the University of Chicago. The Grx3/Fra1-Fra2 plasmids pRSF-Duet1-Fra1His₆/Fra2 and pET21a-Grx3, were created previously in our lab by Dr. Haoran Li (21).

Table 2.1 Primers used for cloning and mutagenesis.

Primer Name	Primer Sequence	Enzyme	Destination Vector
Yap5-For NcoI	5'-GCAG CCATGG CTCTACCTCTGATAAAACC-3'	NcoI	pRSF-DuetI-Yap5
Yap5-Rev EcoRI	5'-GCG AATT CTCTCTTCAGTGGATGAT-3'	EcoRI	pRSF-DuetI-Yap5
Yap5_S116 M_NcoI_F	5'- GCCTCCAAAATGAACTTCAAGCGAAAG CCATG G AAAATCATGCCC-3'	NcoI	pRSF-DuetI-Yap5Δ1-116
Yap5_S116 M_NcoI_R	5'- GGGCATGATTTT CCATGG CTTTCGCTTGAAGTTC ATTTTGGAGGC-3'	NcoI	pRSF-DuetI-Yap5Δ1-116
Yap5_T16 1M_NcoI_F	5'- GCTGGTGGCGCTTGATATG CCATGG TGTACTTAA TAGGTATCGC-3'	NcoI	pRSF-DuetI-Yap5Δ1-161
Yap5_T16 1M_NcoI_R	5'- GCGATACCTATTAAGTAC CCATGG CTATCAAGC GCCACCAGC-3'	NcoI	pRSF-DuetI-Yap5Δ1-161

*Enzyme restriction sites are shown italicized and bold.

Protein Expression and Purification. [2Fe-2S]-Yap5 was prepared as follows. Yap5 expression plasmids were transformed into *E. coli* strain BL21(DE3) and plated on LB agar plates containing 30 µg/mL kanamycin and allowed to grow overnight at 37 °C. These cells were further grown in 1 L cultures, 6 L total, at 37 °C with shaking to an O.D.₆₀₀ 0.6~0.8 and induced with 1 mM isopropyl β-thiogalactoside (IPTG). After induction cells were grown for 18 hours at 30 °C with shaking. Cells were harvested by centrifugation and re-suspended in 50 mL of 50 mM Tris-HCl pH 8.5. A sufficient amount of Pierce Protease Inhibitor Tablets, EDTA-free (Thermo Scientific) was added and cells were lysed via intermittent sonication at 50% amplitude for 10 min pulse time with 40 sec between pulses. The lysate was cleared by centrifugation and filtered through a 0.45 µm polyethersulfone membrane sterile syringe filter (VWR international). The cleared and filtered lysate was then loaded onto a HiTrap QFF anion exchange column (GE Health Care) pre-equilibrated with 50 mM Tris-HCl pH 8.5 and eluted with a linear gradient of 0 to 1 M NaCl. The fractions containing Yap5 as evident by SDS-PAGE were pooled together and concentrated to 2-5 mL. Initially, the protein was then adjusted to 1 M (NH₄)₂SO₄ and loaded onto a Phenyl-Sepharose column (GE Healthcare) equilibrated with 50 mM Tris-HCl pH 8.5, 150 mM NaCl, and 1 M (NH₄)₂SO₄. The protein was then eluted off with a decreasing linear gradient from 1 M to 0 (NH₄)₂SO₄. The fractions containing Yap5 as determined by SDS-PAGE analysis were then pooled and buffer exchanged into 50 mM Tris-HCl pH 8.5, 150 mM NaCl. This step in the procedure was later changed to facilitate cluster transfer studies by the following procedure. After the QFF anion exchange column (GE Healthcare), the fractions containing Yap5 were buffer exchanged into 50 mM Tris pH 7.4, 300 mM NaCl, and loaded onto a 20 mL HiPrep

16/10 Heparin column (GE Healthcare) equilibrated with 50 mM Tris-HCl pH 7.4, 300 mM NaCl. Yap5 was eluted with an isocratic flow of 50 mM Tris-HCl pH 7.4, 300 mM NaCl, pooled and concentrated to 2~3 mL. The protein was then loaded onto a HiLoad Superdex 75 gel filtration column (GE Healthcare) equilibrated with 50 mM Tris-HCl pH 7.4, 300 mM NaCl and eluted in an isocratic flow. The purest fractions of Yap5 as determined by SDS-PAGE analysis were then pooled and concentrated. All of the purifications of Yap5 were performed both aerobically and anaerobically however no significant change in the protein was noted.

Aft2 purification was performed as described previously (21). However, some optimization was carried out as follows: Aft2 expression plasmids were transformed into BL21(DE3) cells, which were grown at 37 °C on LB containing 30 µg/mL kanamycin overnight. Overnight cultures were then inoculated and used for further growth in 1L cultures, 6L total. When the cultures reached an O.D.₆₀₀ 0.6~0.8, they were induced with 1 mM IPTG and incubated at 20 °C for 18 hours. Cells were collected by centrifugation and re-suspended in 50 mL of 50 mM Tris-HCl pH 7.4, 300 mM NaCl. A sufficient amount of Pierce Protease Inhibitor Tablets, EDTA-free (Thermo Scientific) was added and cells were lysed via intermittent sonication. A final amount of 2% streptomycin sulfate was added to precipitate DNA and the lysate was cleared by centrifugation and filtered through a 0.45 µm polyethersulfone membrane sterile syringe filter (VWR International). The cleared lysate was then loaded onto a 20 mL HiPrep 16/10 Heparin column (GE Healthcare) equilibrated with 50 mM Tris-HCl pH 7.4, 300 mM NaCl and eluted with a linear gradient of 300 mM to 1 M NaCl. It was noted through SDS-PAGE that the efficiency of the Heparin column to effectively bind Aft2 was around 50% so the

flow through was collected and ran through the same procedure mentioned above twice to yield a greater amount of protein. After the Heparin column, all fractions containing Aft2 as evident by SDS-PAGE were pooled and concentrated to 1-mL aliquots. The concentrated samples were then loaded onto a HiLoad Superdex 75 gel filtration column (GE Healthcare) equilibrated with 50 mM Tris-HCl pH 7.4, 300 mM NaCl. The protein was eluted off in an isocratic flow and the purest fractions as evident from SDS-PAGE, were collected and concentrated to 1 mL. It is noted here that all buffers initially contained 5 mM dithiothreitol (DTT), this has been previously shown to interfere with cluster transfer studies and so 5 mM Tris[2-carboxyethyl] phosphine (TCEP) was included instead of DTT order to ascertain the validity of the transfer.

Purification of [2Fe-2S] Grx3-Fra2 complex was accomplished via methods previously established (21).

Preparation of Proteins for Fe-S Cluster Transfer. In order to obtain the optimal amount of holo Aft2 it was previously shown that chemical reconstitution was insufficient and so a cluster transfer from Grx3/Fra2 to Aft2 was performed in the following manner (21). In a glove box (Coy Laboratory Products) under anaerobic conditions ($O_2 < 5\text{ppm}$), purified Aft2 20 mg/mL in 1 mL was loaded onto a 5-mL HiTrap desalting column (GE Healthcare) equilibrated with 50 mM Tris-HCl pH 7.4, 300 mM NaCl and eluted with the same buffer to remove any TCEP present. Cluster bound Grx3/Fra2 50 mg/mL in 1 mL was buffer exchanged into 50 mM Tris-HCl pH 7.4, 300 mM NaCl. The proteins were then mixed in a molar ratio of roughly 1:4 and allowed to incubate for 30 min on ice until the transfer was over as monitored by CD spectroscopy. After cluster transfer the mixture was loaded onto a 5-mL Heparin column equilibrated

with 50 mM Tris-HCl pH 7.4, 300 mM NaCl. Grx3/Fra2 was eluted with an isocratic flow of 50 mM Tris-HCl pH 7.4, 300 mM NaCl. Holo Aft2 was eluted via a linear gradient of 300 mM to 1 M NaCl. All fractions containing Aft2 were then collected and concentrated and kept for further study.

Interaction studies on Aft2 and Yap5. For examining the interaction among the apo proteins, concentrations were kept at 400 μ M for each protein in 300 μ L reactions. All samples were prepared in 50 mM Tris-HCl pH 7.4, 300 mM NaCl in the glove box under anaerobic conditions and incubated on ice for 30 min followed by analytical gel filtration chromatography and spectroscopic analysis.

Biochemical and Spectroscopic Methods. Protein concentrations were determined by Bradford assay using BSA as a standard. Iron concentrations were determined using the ferrozine assay (23). Acid labile sulfur concentrations were determined using previously published methods (22). Analytical gel filtration analyses were performed on a Superdex 200 10/300 GL column (GE Healthcare) pre-equilibrated with 50 mM Tris-HCl pH 7.4, 300 mM NaCl. Molecular weights were calculated using a gel filtration calibration kit (GE Healthcare Life Sciences). UV-visible absorption spectra were recorded using a Shimadzu UV 1800 spectrophotometer and a Jasco J-815 polarimeter.

2.4 Results

Yap5 purifies with an Fe-S cluster. In order to characterize the interaction between Aft2 and Yap5, they were first purified individually in order to ascertain their as-purified native states. Yap5 expression trials were initially run on the full-length protein. Several constructs were created for expression in *E. coli* including; native, codon optimized, and His-tagged versions but this proved fruitless as all proteins failed to

express. However, two truncated versions of the protein were generated by genetic engineering by introducing a start site at position 162 in the Yap5 Δ 1-161 mutant or a start site at position 117 in the Yap5 Δ 1-116 mutant that effectively truncated the DNA binding domain of the native protein. The domain structure of these constructs is illustrated in Figure 2.2. Both truncated versions of the protein expressed well as evident from SDS-PAGE analysis (Figure 2.2). However, it was noted that the shorter version (Yap5 Δ 1-161) was more stable and was subsequently used for the majority of the experiments performed. It was noted at the time of purification that Yap5 was reddish brown in hue. UV-visible absorption data further indicated that the Yap5 sample exhibits absorbance maxima at 420 nm and 430 nm (Figure 2.2) which are indicative of a [2Fe-2S]²⁺ cluster (25, 26). CD spectroscopy also indicated spectra consistent with a [2Fe-2S]²⁺ cluster and directly confirmed recent data from another laboratory (18, 26). Iron and acid labile sulfur assays were performed on pure protein samples in triplicate and indicated 1.9 ± 0.2 Fe and 2.1 ± 0.2 sulfurs per homodimer which suggests a final stoichiometry of one [2Fe-2S] cluster per homodimer. This result is in contrast to recently published data (18), where 2 [2Fe-2S] clusters were found to be present. This result could either result from chemical reconstitution or it could be plausible that native inactive Yap5 binds a [2Fe-2S] cluster and a second cluster is required for activation.

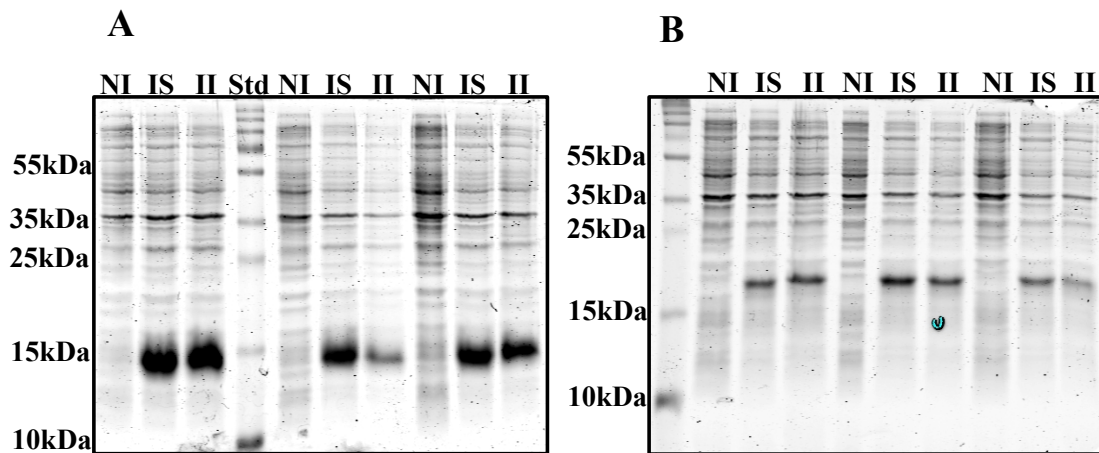


Figure 2.1 SDS-PAGE expression analysis of constructs used in this study. A. SDS-PAGE expression analysis of Yap5 Δ 1-161. NI = non induced cells, IS = Induced soluble, II = Induced Insoluble, STD = Standard. Yap5 Δ 1-161 as a monomer has a theoretical molecular mass of 9.5kDa. **B** SDS-PAGE expression analysis of Yap5 Δ 1-116. Yap5 Δ 1-116 as a monomer has a theoretical molecular mass of 14.5kDa

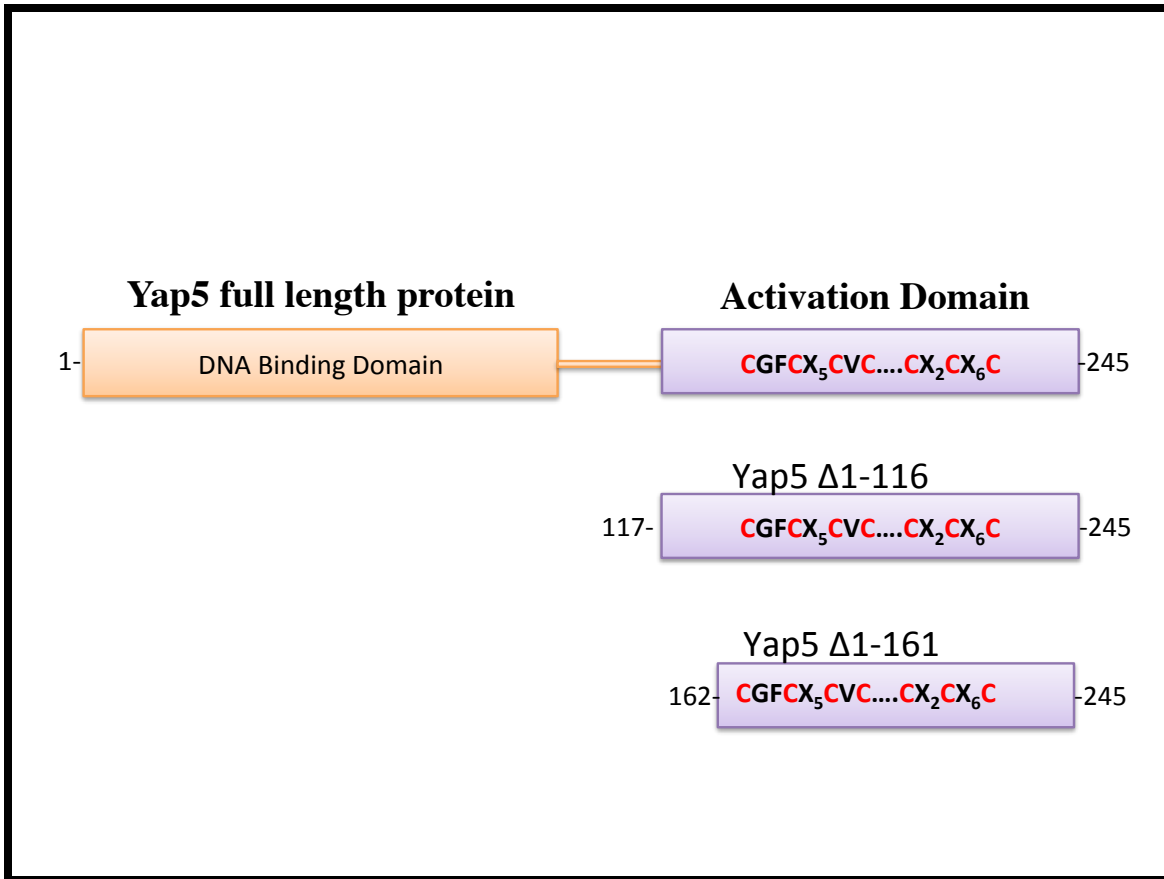


Figure 2.2 Domain structure of Yap5 constructs used in this study. DNA binding domain is shown in tan. Activation domain shown in purple with conserved cysteine residues labeled. Numbers correspond to amino acid sequence.

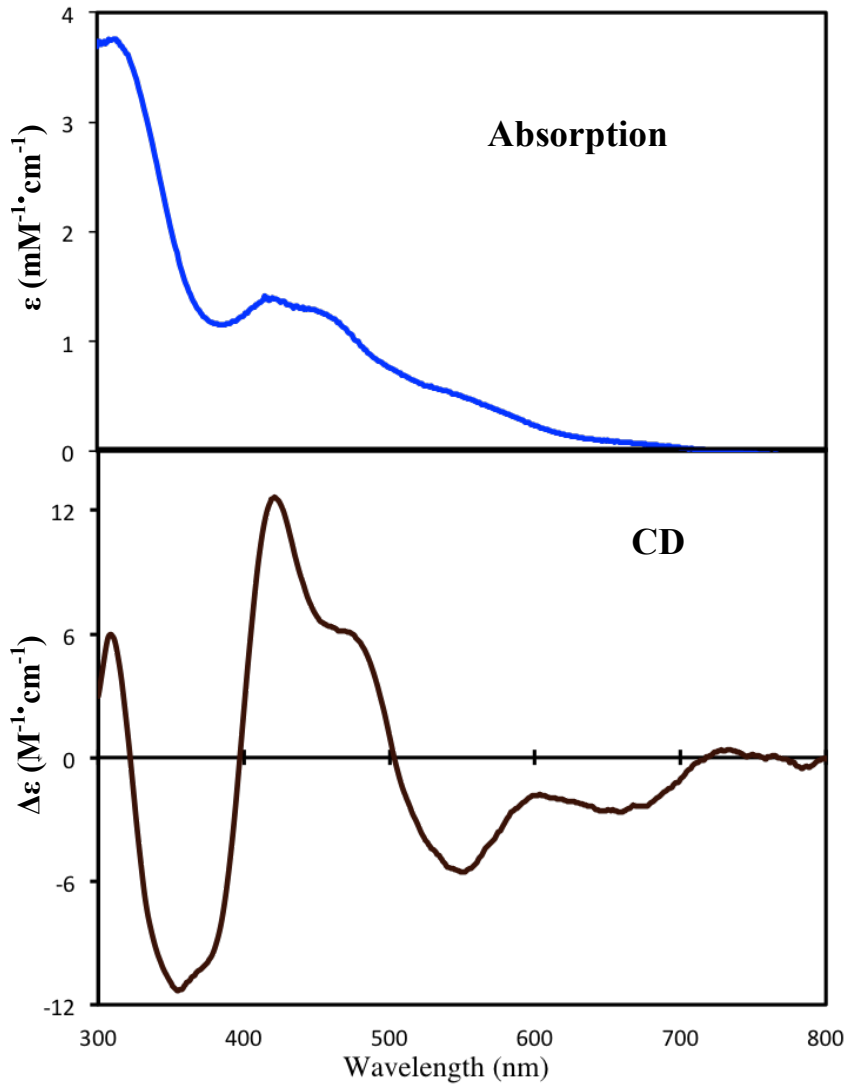


Figure 2.3 UV-visible absorption and CD spectroscopy of Holo Yap5. Spectra were recorded under anaerobic conditions in a sealed 0.1 cm cuvette for [2Fe-2S] Yap5 (300 μ M in homodimer) in 50 mM Tris-HCl with 150 mM NaCl at pH 8.5. $\Delta\epsilon$ and ϵ are normalized to [2Fe-2S] concentration

In Vitro Interaction Studies on Yap5 and Aft2. A genome-wide yeast two hybrid study suggests that Aft1/2 interacts with Yap5 in vivo (19); however, it is not clear if this interaction is physiologically relevant. To address this issue, we mixed holo forms of Aft2 with apo forms of Yap5 and ascertained complex formation by analytical gel filtration chromatography. Surprisingly we found that the two proteins form a stable interaction and co-elute with a higher molecular mass indicating a complex formation (Figure 2.3). Next we tested whether interaction between [2Fe-2S] Aft2 and Yap5 also affected the coordination environment of the cluster. To accomplish this [2Fe-2S] Aft2 was incubated with Yap5 anaerobically and the cluster environment was monitored by UV-visible CD spectroscopy. Even more intriguing was the dramatic change in the CD spectrum from a predominant [2Fe-2S] Aft2 environment when incubated with apo Yap5 (Figure 2.4). The spectral signature clearly changes from holo Aft2 to a predominant [2Fe-2S] Yap5 environment that mirrors the CD spectrum of as purified [2Fe-2S] Yap5. This spectral change is attributed to changes in the cluster coordination environment and suggests cluster transfer from Aft2 to Yap5.

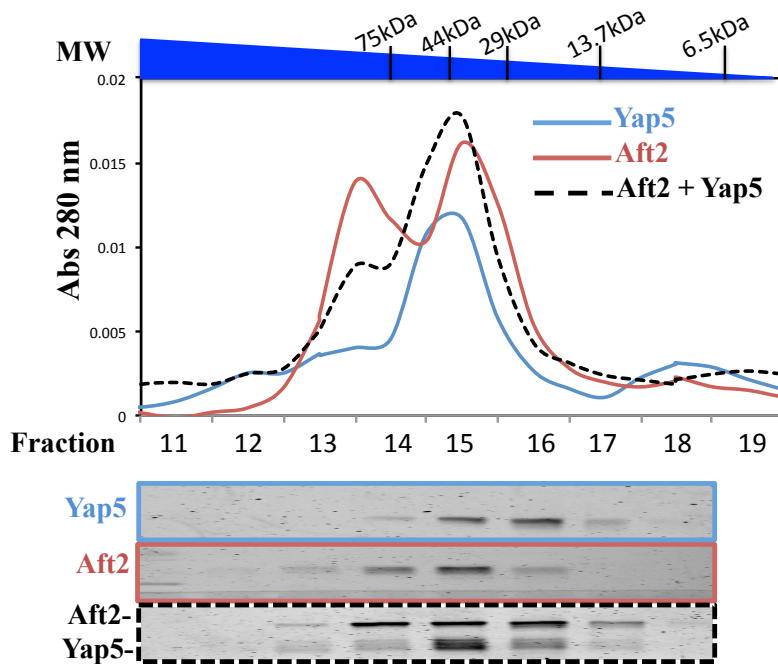


Figure 2.4 Analytical gel filtration chromatography following interaction of Aft2 and Yap5 *Top:* Superdex 200 analytical gel filtration chromatograms of Yap5 (blue), Aft2 (red), and Aft2 +Yap5 in (black dotted). The concentrations of all proteins were kept constant at 200 μ M. The theoretical molecular mass of Aft2 dimer and monomer are 46.6 kDa and 23.3 kDa, respectively, and Yap5 dimer theoretical molecular weight is 29.1 kDa. *Bottom:* SDS-PAGE analysis of all fractions collected.

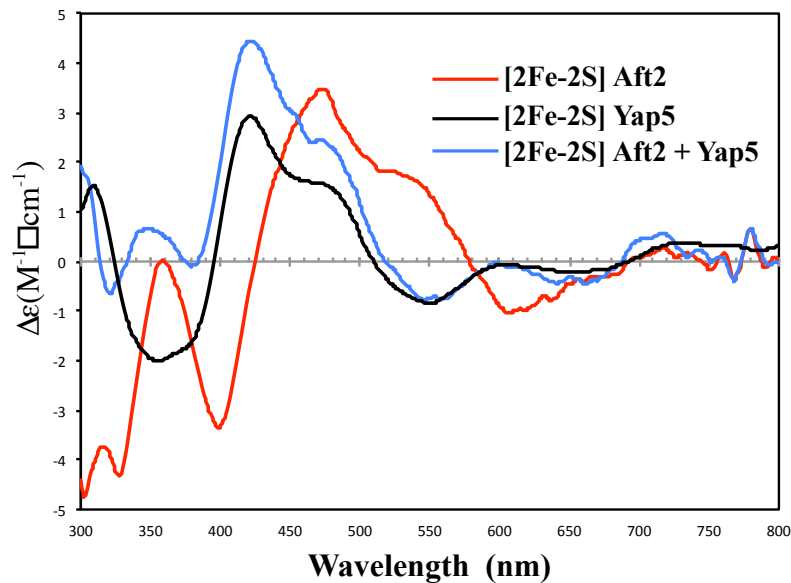


Figure 2.5 CD spectra comparison of [2Fe-2S] Aft2 and Yap5. All protein concentrations were kept constant at 150 μM . [2Fe-2S] Aft2 was incubated with apo-Yap5 for 15 min anaerobically prior to obtaining spectra. $\Delta\epsilon$ is normalized to homodimer concentration.

Yap5 Interacts Directionally and Specifically with [2Fe-2S] Aft2. Our previous data indicates that an Fe-S cluster from [2Fe-2S] Aft2 transfers to Yap5 but it may be that Yap5 is promiscuous in its nature and that the cluster transfer was bidirectional or was interchangeable between itself and other proteins. To test this theory we incubated as-purified [2Fe-2S] Yap5 with apo-Aft2 in a ratio of 2:1 molar excess of [2Fe-2S] Yap5 to drive the transfer forward and monitored CD signal changes. Interestingly, no spectral change in CD signal was noted (Figure 2.5) with the predominant signal [2Fe-2S] Yap5 remaining. This suggests that the direction of the transfer is unidirectional with the cluster transfer proceeding in the direction of [2Fe-2S] Aft2 to apo-Yap5. The question still remained if this was physiologically relevant because Yap5 might be able to acquire a cluster from multiple proteins. In order to answer this question we chose to incubate Yap5 with a control protein Grx3. This protein was chosen because it has previously been shown to interact with Aft2 (12, 21) and would make an interesting addition to the details of iron homeostasis if interactions were seen. We incubated apo-Grx3 with a 2-fold molar excess of [2Fe-2S] Yap5 and monitored CD signal. Strikingly no CD signal change was noted with the predominant signal of [2Fe-2S] Yap5 remaining (Figure 2.5). This result bolsters the conclusion that Yap5 and Aft2 interact specifically with each other and that cluster exchange between the two is likely the mechanism of interaction.

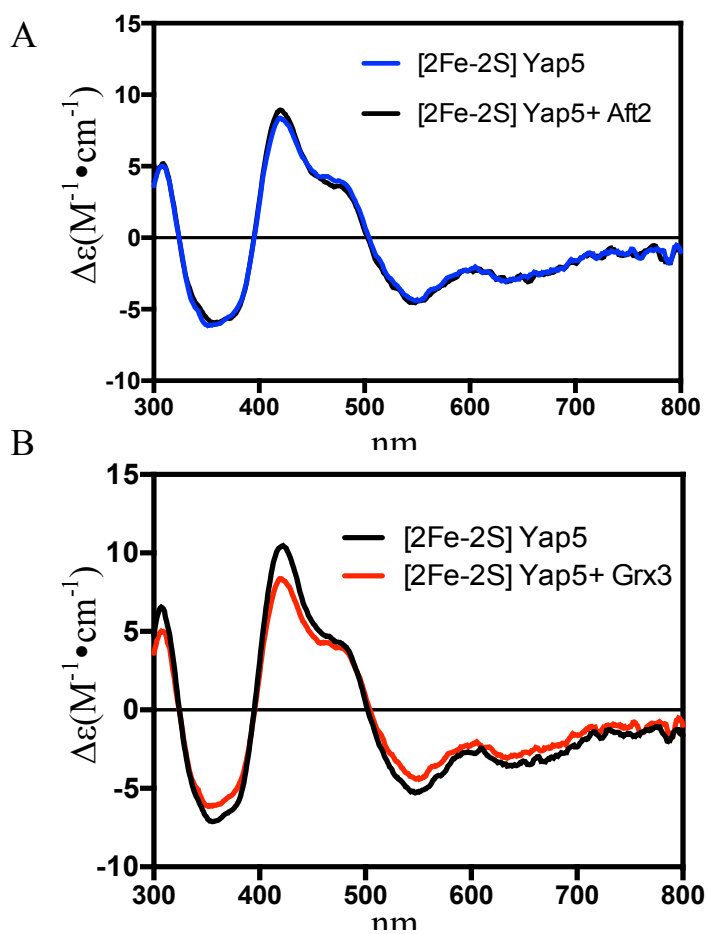


Figure 2.6 CD spectra comparison of [2Fe-2S] Yap5 with apo-Aft2 (A) and (B) [2Fe-2S] Yap5 with apo-Grx3. [2Fe-2S] cluster concentration was kept constant at 300 μ M, while Aft2 or Grx3 ratio was 1:4. $\Delta\epsilon$ values are normalized to [2Fe-2S] concentration.

2.5 Discussion

While the mechanism for Fe-S dependent regulation of the high affinity iron sensors Aft1 and Aft2 have been readily studied, few studies have addressed the low affinity iron sensor Yap5. In this research we sought to investigate the mechanism by which Yap5 senses and regulates intracellular iron. We focused on the interaction between Aft2 and Yap5 in order to study potential crosstalk between these two Fe-S binding transcriptional regulators at the molecular level. We were able to purify truncated versions of Yap5 to gain insight into the interaction between the low and the high iron sensors.

Purified Yap5 exists as a dimer and spectroscopy, iron, and sulfur assays corroborate previous studies that Yap5 harbors a [2Fe-2S] cluster (18). Minor discrepancies do exist, with previous research indicating that Yap5 harbors two distinct [2Fe-2S] clusters and furthermore can tolerate a [4Fe-4S] cluster. This was not observed in our preparation but could be attributed to chemical reconstitution procedures, whereas our spectroscopy and assays were performed on samples as purified. The more interesting story is that Yap5 interacts directly with Aft2 via an Fe-S cluster transfer *in vitro*. Using analytical gel filtration and CD spectroscopy we have shown that Aft2 transfers a cluster to Yap5 *in vitro*. This result is specific to the two proteins and only proceeds in one direction, with transfer of Fe-S cluster from Aft2 to Yap5. This finding if substantiated, would not be without repercussions. In mammalian cells posttranscriptional regulation of both iron sequestration and storage proteins proceeds via an Fe-S signal. (14). If the two transcriptional activators prove to interact through iron Fe-S cluster transfer than our current understanding of mammalian cells could be vastly improved.

This work is not without unknowns, for instance these findings need to be confirmed in vivo in order to be concrete. Although Aft1 and Yap5 were reported to have a physical interaction previously (19), this was conducted using a high throughput yeast two hybrid on non-native proteins. Another striking anomaly is that previous research has shown that the deletion of Grx3/4 does not affect Yap5 activation (18). This is in direct conflict with our model figure 2.7, whereby Grx3/4 are required for [2Fe-2S] cluster delivery to Aft1/2. If Aft1/2 obtains a Fe-S cluster from Grx3/4 which is in turn transferred to Yap5 why then is Yap5 activation still occurring? This is most likely explained by the fact that even when *YAP5* is deleted there is still transcriptional activation of Ccc1 and other genes under Yap5 control, indicating that Yap5 is not the only transcriptional activator, with cells providing alternate methods for iron sequestration.

We are currently in the process of carrying out the task of in vivo research using a wide array of techniques. Currently we are working on a genetic screen utilizing real time PCR and a Aft1/2 double deletion strain in order to ascertain if Yap5 activation still occurs in the absence of Aft1/2 but in the presence of high iron. We are also working on generating bimolecular fluorescence constructs of both proteins, as well as affinity labeled proteins to understand whether our observed phenomena are accurate. If our previous data is confirmed it will not be without significance. The finding that the two iron sensors interact directly with one each other and that the same signal that is the switch of inactivation for one and activation for the other is actually transferred between the two would be a major breakthrough in our understanding of iron homeostasis at the molecular level.

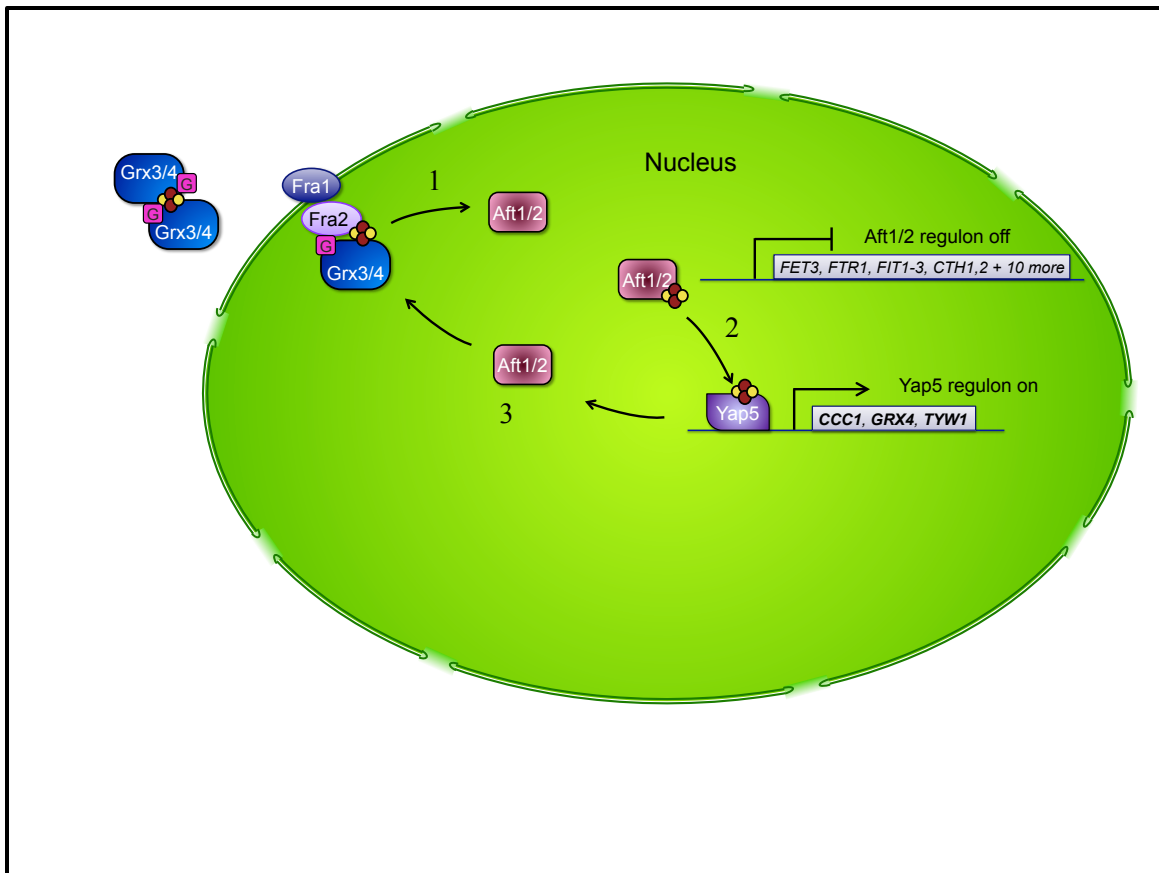


Figure 2.7 Model for Aft2 and Yap5 Interaction. 1. When iron levels are high inside the cell Grx3/4 Fra1/2 complex delivers an inhibitory Fe-S cluster to Aft1/2. 2. Aft1/2 deliver this cluster to Yap5 leading to activation. 3. Elevated levels of Fe-S cluster bound Grx3/4 Fra1/2 complex deliver cluster back on apo-Aft1/2.

CHAPTER 3

SUPPLEMENTARY MATERIALS AND METHODS

Current In Vivo Work While our current data suggest that Aft2 and Yap5 interact in vitro this work needs to be confirmed in vivo. In order to accomplish this task, we have started constructing several genetic mutants for screening the interaction in vivo. First we wanted to create an *AFT1/2* double deletion strain to illuminate if Yap5 activation was still possible in the presence of high iron but in the absence of Aft1/2 via real time PCR. This double deletion strain was accomplished by the following methods. First a pRS405-Aft1 Δ plasmid was created by amplifying *AFT1* sequences from -608 to +20 and +1883 to +2701 using primers included in Table 3.1; which introduced an *NcoI* site at -593, a *BamHI* site at +11, a *PstI* site at +1887, and an *NcoI* site at +2690. The PCR products were then digested at these sites and ligated in a tri-molecular reaction into the *PstI* and *BamHI* sites of the *LEU2* integrating vector pRS405 (27). The resulting pRS405-Aft1 Δ plasmid was verified to be correct by restriction digest and double stranded DNA sequencing (GeneWiz). The pRS405-Aft1 Δ plasmid was linearized with *NcoI* and transformed into the *S. cerevisiae* strain BY4741 *aft2 Δ (Research Genetics) to generate the double deletion strain (BY4741 *aft2 Δ ::*kanMX4* *aft1* Δ ::*LEU2*) by electroporation. All gene deletions, including those that came from Research Genetics were verified to be correct by PCR colony screening, using the Primers included in Table 3.1.**

Table 3.1 Primers used for cloning and colony screening.

Primer name	Primer Sequence	Cut Site	Destination Vector
Aft1Δ-Up-Fwd	5'-CGGAAAATGCCATGGAGATGAGTC-3'	NcoI	pRS-405 AFT1Δ
Aft1Δ-Up-Rev	5'-GCCGGATCCAAGCCTTCCATTG-3'	BamHI	pRS-405 AFT1Δ
Aft1Δ-Down-Fwd	5'-GCCACTGCAGCAATTGCAATATC-3'	PstI	pRS-405 AFT1Δ
Aft1Δ-Down-Rev	5'-GCAGTCCATGGGTCTACAGG-3'	NcoI	pRS-405 AFT1Δ
Aft1-A-WT	5'-AGCAGAAACAGAATTTCGCATATTAC-3'	N/A	Screening Aft1
Aft1-B-WT	5'-CTTATCTTCAAAGTTGGGTACTGGA-3'	N/A	Screening Aft1
Aft1-C-WT	5'-GCAACTCACTTTTAAGACAAGAAGC-3'	N/A	Screening Aft1
Aft1-D-WT	5'-CAA AATTAATGACAGAGGGAGAGAA-3'	N/A	Screening Aft1
Aft1Δ-C1	5'-GACAGGTATCCGGTAAGC-3'	N/A	Screening Aft1
Aft1Δ-B1	5'-GTGAACCATCACCTAATCAAG-3'	N/A	Screening Aft1
Aft2-A-WT	5'-CTTCTTCACTTTAACCTGTCTGAGC-3'	N/A	Screening Aft2
Aft2-B-WT	5'-ATAATTGGTGTGACGAGTGGTAAGT-3'	N/A	Screening Aft2
Aft2-C-WT	5'-ATCTTCACCAAATTTTATGGAAACA-3'	N/A	Screening Aft2
Aft2-D-WT	5'-TTTTTAGATAATTGAATGTTGCGGT-3'	N/A	Screening Aft2
Aft2Δ-kanB1	5'-TGTACGGGCGACAGTCACAT-3'	N/A	Screening Aft2
Aft2Δ-KanC3	5'-CCTCGACATCATCTGCCAGAT-3'	N/A	Screening Aft2

*Enzyme restriction sites are shown italicized and bold.

REFERENCES

1. Rodrigues-Pousada, C.; Menezes, R. A.; Pimentel, C., The Yap family and its role in stress response. *Yeast* **2010**, *27* (5), 245-58.
2. Outten, C. E.; Albetel, A. N., Iron sensing and regulation in *Saccharomyces cerevisiae*: Ironing out the mechanistic details. *Curr Opin Microbiol* **2013**, *16* (6), 662-8.
3. Imlay, J. A., Iron-sulphur clusters and the problem with oxygen. *Molecular microbiology* **2006**, *59* (4), 1073-82.
4. Halliwell, B., Role of free radicals in the neurodegenerative diseases: therapeutic implications for antioxidant treatment. *Drugs & aging* **2001**, *18* (9), 685-716.
5. Chitambar, C. R.; Kotamraju, S.; Wereley, J. P., Expression of the hemochromatosis gene modulates the cytotoxicity of doxorubicin in breast cancer cells. *International journal of cancer. Journal international du cancer* **2006**, *119* (9), 2200-4.
6. Meneghini, R., Iron homeostasis, oxidative stress, and DNA damage. *Free radical biology & medicine* **1997**, *23* (5), 783-92.
7. Kassebaum, N. J.; Jasrasaria, R.; Naghavi, M.; Wulf, S. K.; Johns, N.; Lozano, R.; Regan, M.; Weatherall, D.; Chou, D. P.; Eisele, T. P.; Flaxman, S. R.; Pullan, R. L.; Brooker, S. J.; Murray, C. J., A systematic analysis of global anemia burden from 1990 to 2010. *Blood* **2014**, *123* (5), 615-24.
8. De Freitas, J.; Wintz, H.; Kim, J. H.; Poynton, H.; Fox, T.; Vulpe, C., Yeast, a model organism for iron and copper metabolism studies. *Biometals : an international*

journal on the role of metal ions in biology, biochemistry, and medicine **2003**, 16 (1), 185-97.

10. Rutherford, J. C.; Ojeda, L.; Balk, J.; Muhlenhoff, U.; Lill, R.; Winge, D. R., Activation of the iron regulon by the yeast Aft1/Aft2 transcription factors depends on mitochondrial but not cytosolic iron-sulfur protein biogenesis. *The Journal of biological chemistry* **2005**, 280 (11), 10135-40.

11. Ueta, R.; Fujiwara, N.; Iwai, K.; Yamaguchi-Iwai, Y., Iron-induced dissociation of the Aft1p transcriptional regulator from target gene promoters is an initial event in iron-dependent gene suppression. *Molecular and cellular biology* **2012**, 32 (24), 4998-5008.

12. Li, H.; Mapolelo, D. T.; Dingra, N. N.; Naik, S. G.; Lees, N. S.; Hoffman, B. M.; Riggs-Gelasco, P. J.; Huynh, B. H.; Johnson, M. K.; Outten, C. E., The yeast iron regulatory proteins Grx3/4 and Fra2 form heterodimeric complexes containing a [2Fe-2S] cluster with cysteinyl and histidyl ligation. *Biochemistry* **2009**, 48 (40), 9569-81.

13. Li, L.; Bagley, D.; Ward, D. M.; Kaplan, J., Yap5 is an iron-responsive transcriptional activator that regulates vacuolar iron storage in yeast. *Molecular and cellular biology* **2008**, 28 (4), 1326-37.

14. Li, L.; Miao, R.; Bertram, S.; Jia, X.; Ward, D. M.; Kaplan, J., A role for iron-sulfur clusters in the regulation of transcription factor Yap5-dependent high iron transcriptional responses in yeast. *The Journal of biological chemistry* **2012**, 287 (42), 35709-21.

15. Pimentel, C.; Vicente, C.; Menezes, R. A.; Caetano, S.; Carreto, L.; Rodrigues-Pousada, C., The role of the Yap5 transcription factor in remodeling gene expression in response to Fe bioavailability. *PloS one* **2012**, 7 (5), e37434.

16. Wood, M. J.; Andrade, E. C.; Storz, G., The redox domain of the Yap1p transcription factor contains two disulfide bonds. *Biochemistry* **2003**, *42* (41), 11982-91.
17. Fernandes, L.; Rodrigues-Pousada, C.; Struhl, K., Yap, a novel family of eight bZIP proteins in *Saccharomyces cerevisiae* with distinct biological functions. *Molecular and cellular biology* **1997**, *17* (12), 6982-93.
18. Rietzschel, N.; Pierik, A. J.; Bill, E.; Lill, R.; Muhlenhoff, U., The basic leucine zipper stress response regulator Yap5 senses high-iron conditions by coordination of [2Fe-2S] clusters. *Molecular and cellular biology* **2015**, *35* (2), 370-8.
19. Uetz, P.; Giot, L.; Cagney, G.; Mansfield, T. A.; Judson, R. S.; Knight, J. R.; Lockshon, D.; Narayan, V.; Srinivasan, M.; Pochart, P.; Qureshi-Emili, A.; Li, Y.; Godwin, B.; Conover, D.; Kalbfleisch, T.; Vijayadamodar, G.; Yang, M.; Johnston, M.; Fields, S.; Rothberg, J. M., A comprehensive analysis of protein-protein interactions in *Saccharomyces cerevisiae*. *Nature* **2000**, *403* (6770), 623-7.
20. Rietzschel, N.; Pierik, A. J.; Bill, E.; Lill, R.; Muhlenhoff, U., The basic leucine zipper stress response regulator Yap5 senses high-iron conditions by coordination of [2Fe-2S] clusters. *Molecular and cellular biology* **2015**, *35* (2), 370-8.
21. Li, H. Characterization and Functional Studies of Fe-S Cluster Binding Monothiol Glutaredoxin-Bola Complexes Regulating Iron Homeostasis. Ph. D. Dissertation, University of South Carolina, Columbia, SC, 2011
22. Broderick, J.B; Henshaw, T. F.; Cheek, J.; Wojtuszewski, K.; Smith, S. R.; Trojan, M. R.; McGhan, R. M.; Kopf, A.; Kibbey, M.; Broderick, W. E., Pyruvate formate-lyase-activating enzyme: strictly anaerobic isolation yields active enzyme containing [3Fe-4S]⁺ cluster. *Biochem Biophys Res Commun* **2000**, *269*, 451-6

23. Reimer, J.; Hoepken, H. H.; Czerwinska, H.; Robinson, S. R.; Dringen, R. Colorimetric ferrozine-based assay for the quantitation of iron in cultured cells. *Anal Biochem* 2004, 331, 370-5
24. Hong Enriquez, R., P.; Do, T., N.; Bioavailability of metal ions and evolutionary adaptation. *Life* 2012, 2, 274-285
25. Dailey, H. A.; Finnegan, M. G.; Johnson, M. K. Human ferrochelatase is an iron sulfur protein. *Biochemistry* 1994, 33, 403-407
26. Stephens, P. J.; Thomson, A. J.; Dunn, J. B. R.; Keiderling, T. A.; Rawlings, J.; Rao, K. K.; Hall, D. O. Circular dichroism and magnetic dichroism of iron-sulfur proteins. *Biochemistry* 1978, 17, 4770-4778
27. Sikorski, R. S.; Heiter, P. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* 1989, 122, 119-27