MOLECULAR MECHANISMS UNDERLYING AEROBIC RESPIRATION AND HEME REGULATION IN YEAST

by

Sneha Lal

APPROVED BY SUPERVISORY COMMITTEE:

_____

Dr. Li Zhang, Chair

_____

Dr. John G. Burr

_____

Dr. Heng Du

_____

Dr. Zhenyu Xuan

This dissertation is dedicated to my pillar of strength, my parents Shri Bachche Lal and Sheela and my dearest brother Brijesh Lal

MOLECULAR MECHANISMS UNDERLYING AEROBIC RESPIRATION AND HEME REGULATION IN YEAST

by

SNEHA LAL, MSc

DISSERTATION

Presented to the Faculty of

The University of Texas at Dallas

in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY IN

MOLECULAR AND CELL BIOLOGY

THE UNIVERSITY OF TEXAS AT DALLAS

December 2017

ACKNOWLEDGEMENTS

This dissertation would not have been possible without the love, faith and support of my teachers, family and friends. I am highly grateful and indebted to my mentor, Dr. Li Zhang who has guided me and extended her support throughout my time at UT Dallas. I am thankful to my dissertation committee members Dr. John Burr, Dr. Heng Du and Dr. Zhenyu Xuan for their valuable input and feedback to complete this dissertation.

I express my deepest gratitude to my loving and caring parents, Shri Bachche Lal and Sheela, and my dearest brother, Brijesh Lal who have immense faith in me. Their undying love and support helped me pursue my studies. I am grateful to my loving husband, Sudhanshu Shekhar, who is an added blessing in my life. I thank my wonderful colleagues Chaitanya Konduri, Dr. Ajit Shah, Tianyuan Wang, Maksudal Alam for being a great team and providing an enthusiastic work atmosphere. I am very thankful to my loving friends Sarada Preeta Kalainayakan, Sanchareeka Dey, Poorva Ghosh, Thai Cao, Chandni Jain, Riddhi Sheth, Harika Nagandla, Sharmila Nair, Purvi Vyas, Aakriti Sharma, Vibhuti Gupta, Salvador Moreno, Vasanth Murali who have always extended their love and support.

October 2017

MOLECULAR MECHANISMS UNDERLYING AEROBIC RESPIRATION AND HEME REGULATION IN YEAST

Sneha Lal, PhD
The University of Texas at Dallas, 2017

Supervising Professor: Dr. Li Zhang

Heme is an important signaling molecule with diverse functions in living organisms ranging from regulating gene transcription to circadian rhythm. In yeast, heme serves as a secondary messenger of oxygen, as heme synthesis depends on the intracellular levels of oxygen. Yeast and higher eukaryotes have adapted to adverse conditions including low oxygen or hypoxia. To understand the molecular mechanisms underlying hypoxia tolerance on a genome-wide scale, protein localization was studied using green fluorescence protein (GFP) tagged library of yeast genes under normoxia and hypoxia by Henke and colleagues. This study identified more than 200 proteins that change their localization under hypoxia. Among them were proteins of the chromatin remodeling SWI/SNF complex. Six proteins of the SWI/SNF complex- Swi3, Snf5, Snf6, Snf11, Snf12 and Swp82 relocalized under hypoxia. One of the objectives of this dissertation was to study the mechanism of regulation of cellular bioenergetics and respiration by the SWI/SNF proteins. Measurement of oxygen consumption rate and promoter activity show that Swi3, not Swi2, regulates aerobic gene expression and oxygen consumption. The levels of mitochondrial respiratory chain complex proteins were found to be increased in *Δswi3* cells as compared to the parent cells. Deletion of *SWI3* also induced the expression of aerobic respiration

genes under high heme conditions. Computational analysis of the promoters of the genes bound by the human homologs of Swi3, BAF155 and BAF170 also show that they modulate aerobic respiration genes. In the next part of the dissertation, we have studied the regulation of a transcription factor Gis1 which was initially identified as a stress response regulator. In the previous genome-wide study of protein sub-cellular localization under hypoxia, Gis1 was identified as one of the fast responders to hypoxia and reoxygenation. Here we have studied the regulation of the transcriptional activity of Gis1 by heme. Gis1 belongs to the JmjC family of histone demethylases. Gis1 protein has two heme regulatory motifs (HRM). We show that the DNA binding zinc finger domain of Gis1 promotes the heme activation of its transcriptional activity, although heme does not affect Gis1 binding to DNA. These results have identified a new class of heme signaling proteins.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

1.1 Heme biosynthesis requires oxygen

Heme, or iron protoporphyrin IX, is an important prosthetic group in numerous hemoproteins as well as cofactor to enzymes. Heme is a planar structure with four pyrrole rings linked by methene bridges and iron chelated at the center. The iron in heme can exist in ferrous (reduced) or ferric (oxidized) state, see Fig. 1.1. All eukaryotes except the parasitic helminths and the nematode Caenorhabditis *elegans* synthesize heme *de novo* [1], although they require heme for existence. Its biosynthesis requires 10 different enzymes [2]. Heme biosynthesis proceeds with a condensation of glycine and succinyl Co-A in the mitochondria which forms 5-aminolevulinic acid (ALA), catalyzed by 5-aminolevulinate synthase (ALAS), which is the rate limiting enzyme [3].

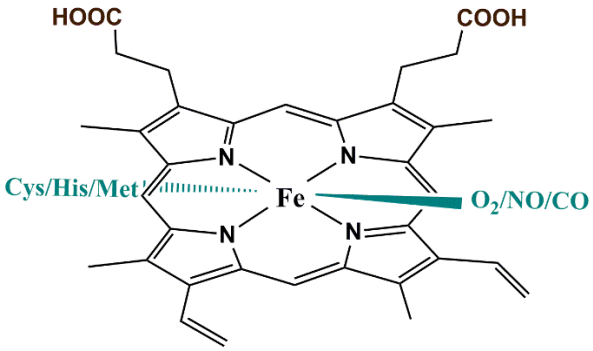


Figure 1.1. The structure of heme (iron protoporphyrin IX)

Higher eukaryotes express two ALA synthases – ALAS1 (ubiquitous) and ALAS2 (erythroid-specific) [4]. ALA reaches the nucleus and an asymmetric condensation of two ALA molecules gives rise to porphobilinogen (PBG) in a reaction catalyzed by porphobilinogen synthase. In the next step, four molecules of PBG are converted to an unstable tetrapyrrole called

hydroxymethylbilane (HMB) by porphobilinogen deaminase (PBGD). HMB is converted to a tetrapyrrole uroporphyrinogen III, by uroporphyrinogen III synthase. Uroporphyrinogen decarboxylase (UROD) catalyzes the removal of the four carboxylic group from the acetic acid side chains, giving rise to coproporphyrinogen III. For the next step, coproporphyrinogen III enters the mitochondria again. Coproporphyrinogen oxidase (CPO) converts coproporphyrinogen III to protoporphyrinogen IX by carrying out oxidative decarboxylation of the propionate groups of the pyrrole rings of coproporphyrinogen III. In the step before last, protoporphyrinogen oxidase (PPO), oxidizes protoporphyrinogen IX to give protoporphyrin IX (PP IX). This step requires oxygen. The final step in the biosynthesis of heme is insertion of iron in the center of PP IX by the enzyme ferrochelatase.

1.2 Intracellular level of heme is regulated

The intracellular heme content must be maintained as excess free heme has toxic effects [5, 6]. Free heme is not reused and must be degraded. Heme homeostasis is maintained by its synthesis, degradation, heme transporters and hemoproteins [7]. The first product of heme synthesis ALA, exerts a negative feedback on the pathway. Heme binds to the HRM on ALAS preventing it from entering the mitochondria, thus blocking heme synthesis. The catabolism of heme is executed by the enzyme heme oxygenase (HO). HO converts heme to biliverdin which is further converted to bilirubin by bilirubin reductase [8]. Hemopexin (Hx), a plasma glycoprotein, binds heme non-covalently with an affinity of K_D 10^{-14}, highest among known heme-binding proteins [9, 10]. Hx mainly expressed in the liver, scavenges excess free heme from the circulating blood. Another mechanism that keeps a check on the heme levels is by Rev-erbα (NR1D1), which belongs to a family of nuclear receptors. Rev-erbα binds heme which

leads to the repression of the transcription factor PGC-1α [11]. PGC-1α is known to upregulate the expression of ALAS1, thereby reducing heme levels.

1.3 The biological role of heme is multifaceted

The function of heme as a prosthetic group in proteins and enzymes involved in the transport, storage, and utilization of oxygen is well-known [12]. Furthermore, heme directly regulates the expression of proteins and enzymes involved in oxygen utilization [13]. In humans, heme plays essential roles in many physiological processes, including erythropoiesis, neurogenesis, cell growth and differentiation [13-15]. Heme constitutes 95% of functional iron in human body, as well as two-thirds of the average person's iron intake in developed countries. In human body, erythroid and hepatic cells use the most heme. Most, if not all, human cells need a basal level of heme for survival. Mammalian cells can synthesize heme endogenously in mitochondria, or they can import heme from the circulation via heme transporters (Fig. 1.2) [[16] and references therein]. In mammalian cells, intracellular heme is used to synthesize various hemoproteins, such as cytochromes, or it can be degraded by heme oxygenase (Fig. 1.2) [17]. Heme serves as a regulatory and signaling molecule and directly regulates transcription, translation, cell growth and differentiation [15]. For example, in erythroid precursor cells, heme regulates the transcription of globin chains and heme oxygenase genes by modulating the activity of transcriptional regulators, such as NF-E2 and Bach1 [18-21]. Additionally, heme regulates the translation of globin chains by directly controlling the activity of the heme-regulated eIF-2α kinase (HRI) [22, 23]. These mechanisms ensure the coordination of globin chain synthesis with heme synthesis.

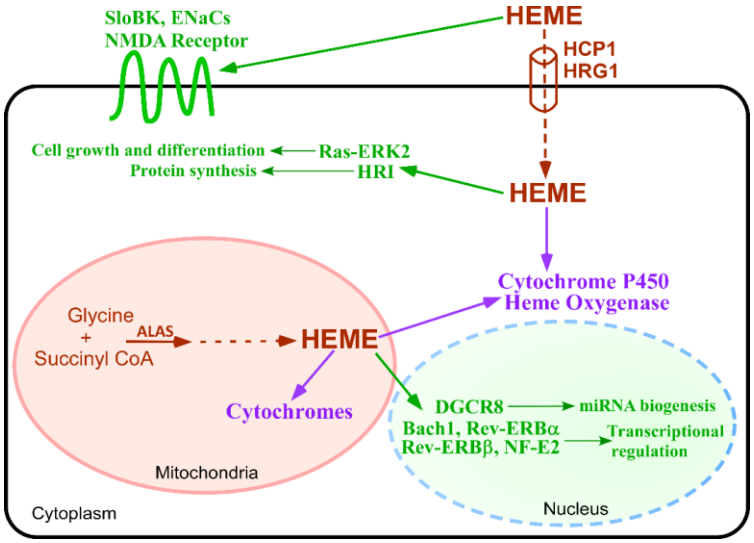


Figure 1.2. The signaling and structural functions of heme in human cells. Human cells can synthesize heme de novo in mitochondria (the first and rate-limiting enzyme is ALAS, 5-aminolevulinate synthase) or import heme via heme transporters, such as HRG1 and HCP1. Inside cells, heme serves as a prosthetic group in numerous enzymes and proteins that transport, store, or use oxygen, such as mitochondrial cytochromes and cytochrome P450. Additionally, heme directly regulates the activity of diverse cellular signaling and regulatory molecules, such as Bach1, Rev-ERBα, and Rev-ERBβ (transcriptional regulators), as well as DGCR8 (an essential miRNA processing factor) in the nucleus. Heme also regulates HRI (the heme-regulated inhibitor kinase controlling protein synthesis) and the Ras-ERK signaling pathway in the cytoplasm. Furthermore, heme regulates the activity of the NMDA receptor, the SloBK potassium channel, and the ENaCs sodium channel on the cell membrane.

In neuronal cells, heme can modulate the activity of the NMDA receptor and the Ras-ERK1/2 signaling pathway [24-27]. Furthermore, heme directly regulates the activity of the nuclear receptors REV-ERBα and REV-ERBβ [28, 29], microRNA processing protein DiGeorge critical region-8 (DGCR8) [30], and ion channels (SloBK potassium channel and epithelial sodium channel ENaCs) [31-33], in an array of mammalian cells (Fig. 1.2). Heme is also involved in the pathogenesis of several types of cancer and diseases. Excessive heme intake through diet intake is linked to higher risk of several cancer types like colorectal, colon, gastrointestinal, pancreatic, endometrial and lung [16]. Thus targeting heme availability could be a novel therapeutic strategy against tumorigenesis. Heme is also linked to increased risk of type-2 diabetes and coronary

heart disease. Defective heme synthesis causes diseases such as anemia and porphyria. Heme is required for the proper functioning of several proteins including the oxidative phosphorylation complexes II, III and IV in the mitochondria. Particularly, multiple subunits in complexes III and IV require heme as a prosthetic group, and different forms of heme are present (Fig. 1.3) [34]. As mitochondria is the site for oxidative phosphorylation (OXPHOS), majority of the oxygen is consumed by mitochondria. Mitochondrial respiration is carried out by the OXPHOS complexes I–V (Fig. 1.3) [35]. Complex I, the NADH-coenzyme Q reductase or NADH dehydrogenase, is constituted of 45 polypeptides, of which seven (ND-1, -2, -3, -4, -4L, -5, and -6) are encoded by mtDNA, and the rest are encoded by nuclear DNA [36, 37]. Complex II, succinate-coenzyme Q reductase or succinate dehydrogenase, contains four nDNA-encoded protein subunits.

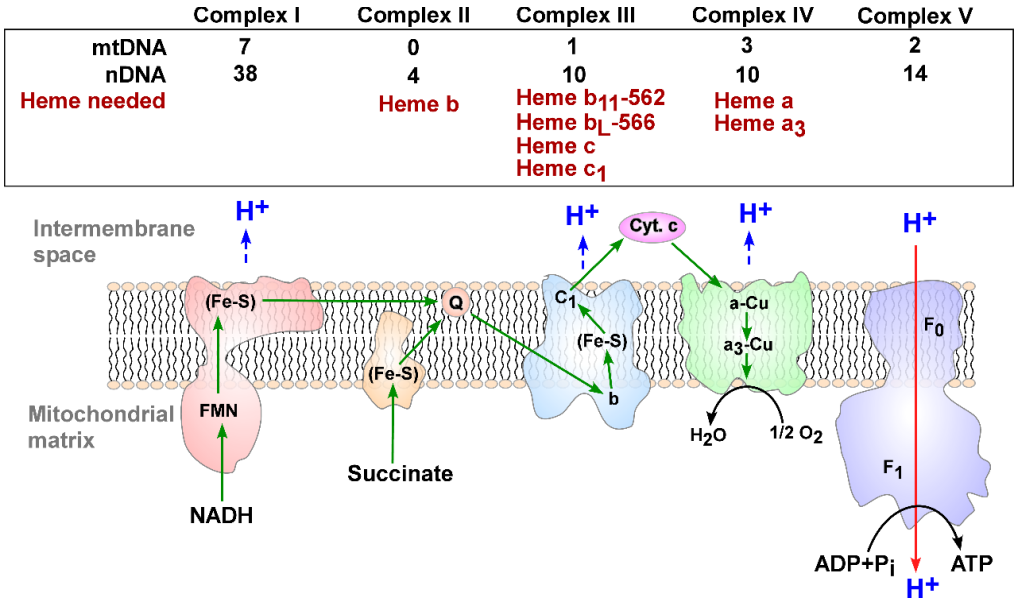


Figure 1.3. The function and composition of mitochondrial OXPHOS complexes I–V. Shown here are the directions of electron and proton transport by the OXPHOS complexes. Also indicated are the origins of DNA encoding the subunits and the hemes needed for complexes II–IV. *nDNA* nuclear DNA, *mtDNA* mitochondrial DNA.

Complex III, cytochrome bc1 complex or ubiquinol-cytochrome c oxidoreductase, contains 11 subunits, of which one (cytochrome b) is encoded by the mtDNA. Complex IV, cytochrome c reductase, is composed of 13 subunits, of which three (CO-I, -II, and -III) are from the mtDNA. Complex V, ATP synthase, contains approximately 16 subunits, of which two (ATP-6 and -8) are from mtDNA. Complexes I, III, IV, and V retain mtDNA-encoded protein subunits and transport protons (Fig. 1.3).

1.4 Hemoproteins function as oxygen sensors

One of the major functions extensively studied about heme is its role in oxygen sensing. Since heme synthesis requires oxygen, heme and hemoproteins serve as oxygen sensors [38]. The ferrous iron of heme binds to the proteins through the amino acids – histidine, tyrosine, or cysteine [39]. Once heme is synthesized in the mitochondria, it is transported to different cellular compartments for its function. Hap1 is a widely studied yeast transcription factor whose transcriptional activity is mediated by heme levels in the cell. Hap1 is 1483 amino acids long and contains several domains [40]. It has a DNA binding domain, a dimerization domain, a heme-responsive domain, and an activation domain. The transcriptional activity of Hap1 is directly proportional to the concentration of heme. This activation of the transcriptional activity of Hap1 is achieved by the binding of heme to a conserved heme regulatory motif – (R/K)CP(I/V)XX in the heme-responsive domain of Hap1. There are seven HRMs in Hap1 and only one HRM/CP motif is required for heme regulation. Another example of a well-studied transcription factor regulated by heme is Bach1. Bach1 is a transcription repressor in mammalian cells which is negatively regulated by heme [41]. Its known target genes are HO-1, ferritin and ferroportin which are involved in heme degradation. Bach1 contains six HRM motifs and they are required

for heme binding to Bach1 which inactivates it. Under normal conditions, Bach1 is known to form heterodimer with MafK and then it binds to the Maf recognition elements (MARE). Upon direct binding of heme to the CP motifs of Bach1, the DNA binding ability is inhibited. Thus, heme negatively regulates the activity of Bach1. Recent studies show that heme also regulates macrophages through the Bach1 pathway [42]. Some other well-studied hemoproteins are cytochrome c, hemoglobin, myoglobin, neuroglobin, cytochrome P450 and cytochrome b5. Microarray gene expression profiling analysis on the genes regulated by heme globally shows that Hap1 regulates only a small fraction of the genes regulated by heme and oxygen [15, 43]. The transcription factors involved in oxygen-sensing in yeast also include Hap2/3/4/5, Mga2, and Rox1. Other key regulators are yet to be identified that are regulated by heme. Recent studies in our lab have identified the role of SWI/SNF chromatin remodeling complex proteins in oxygen regulation.

1.5 The conserved regulatory SWI/SNF complex proteins mediate oxygen regulation and hypoxia response

SWI/SNF is a chromatin remodeling complex that is highly conserved in eukaryotes from yeast to human. Recent protein localization studies have shown that SWI/SNF complex proteins play an important role under hypoxia. There are two classes of chromatin remodelers – (i) ATP-dependent complexes and (ii) histone acetyltransferase (HAT) and histone deacetylase (HDAC) complexes [44]. The SWI/SNF complex belongs to the former class of chromatin remodeling. For transcription of genes, DNA replication and repair, chromatin structure undergoes rearrangements which is facilitated by chromatin remodelers. ATP dependent chromatin

remodeling complexes utilize the energy of ATP hydrolysis to locally modify or disrupt histone DNA association [45]. Purified SWI/SNF complex contains about 10-12 polypeptides. It has an apparent molecular weight of 1.14 MDa in yeast and about 2 MDa in higher eukaryotes [46]. Swi3, a part of this complex, controls SWI/SNF assembly, ATP dependent H2A-H2B displacement and recruitment to target genes. A wide array of yeast genes, about 5-7% of the total yeast genome is regulated by SWI/SNF proteins [47-49]. Swi3 and its human homologues contain three conserved domains- the SWIRM domain, SANT domain and leucine-zipper motif [46]. Mutations in one or several components of the mammalian SWI/SNF are associated with various types of cancer [50, 51]. The human homolog of Swi3, BAF155 has been identified as a human tumor suppressor gene. The GO and KEGG analysis of the gene targets of SWI/SNF in the human genome was also found to be overrepresented for several types of cancers such as pancreatic cancer and chronic myeloid leukemia [52]. This indicates that the targets of SWI/SNF complex proteins also serve an important function in different cancer pathways.

Protein localization study on a genome-wide scale in yeast showed that 203 proteins redistribute under hypoxia of which more than 120 nuclear proteins are retained in the cytoplasm [53, 54]. Interestingly, six nuclear proteins belonging to the SWI/SNF complex – Swi3, Snf5, Snf6, Snf11, Snf12 and Swp82 were retained in the cytoplasm under hypoxia (Fig. 1.4). Swi3, Snf6 and Swp82 redistributed under hypoxia and reoxygenation in a short period of time, implying a functional role in initiating downstream events such as gene expression in response to hypoxia [54]. This data conforms with the computational analysis of the genes regulated by SWI/SNF in the presence and absence of Hap1 (Table 1.1) [54].

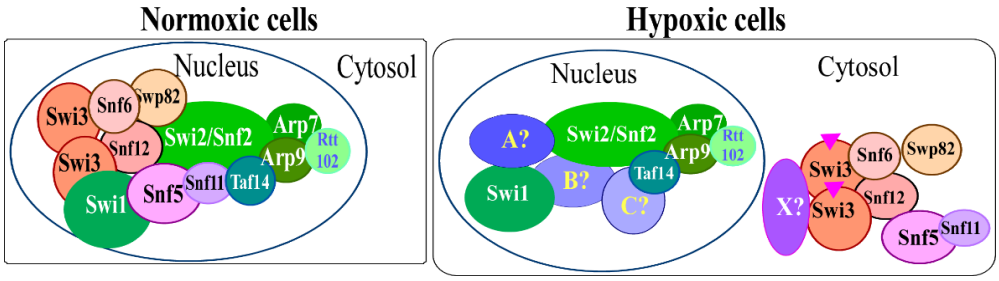


Figure 1.4. A cartoon illustrating how oxygen may affect SWI/SNF composition and function. In normoxic cells, the components form the SWI/SNF complex in the nucleus, enabling it to remodel chromatin at the target genes. In hypoxic cells, Swi3 and five other components are retained in the cytosol, perhaps due to modifications of these components and/or interactions with unidentified factor(s) X. In the nucleus, Swi2 and other remaining components may interact with some other proteins (marked as A, B, and C), forming complexes with different composition and targeting different sets of genes [54].

The number of targets altered by SWI/SNF under hypoxia were similar in presence or absence of Hap1, indicating an important and independent role of SWI/SNF complex proteins in mediating oxygen regulation and hypoxia response.

Table 1.1. The number of Swi/Snf targets whose transcript level is regulated by oxygen [54].

	HAP1 cells		Δ *hap1* cells	
	Targets	**p-value**	**Targets**	**p-value**
Swi2	95	7.55E-69	119	2.87E-69
Swi3	112	6.14E-94	118	7.31E-10
Snf5	67	5.24E-49	71	1.05E-53
Snf6	109	8.06E-73	139	1.10E-10
Snf11	9	6.79E-10	6	7.45E-06
Taf14	19	8.09E-19	20	3.0E-20

We further probed the role of Swi3 complex proteins in aerobic respiration (see Chapter 2). We found that Swi3 suppresses aerobic respiration genes and oxygen consumption. The human homologues of Swi3, BAF155 and BAF170 also exhibited increased rate of oxygen consumption. add a sentence on HIF. In mammalian cells, the hypoxia-inducible factor HIF-1α,

is a target of the SWI/SNF complex [55]. The cellular response to hypoxia in higher eukaryotes is mainly mediated by the transcription factor HIF-1α. BAF57, a component of SWI/SNF targets the complex to HIF-1α promoter under hypoxia. These results demonstrate that SWI/SNF complex is involved in oxygen sensing and aerobic respiration.

1.6 The regulator HIF-1 mediates cellular adaptive response to hypoxia

Oxygen is vital for the existence of all aerobic organisms. A sufficient amount of oxygen is required for the synthesis of ATP through mitochondrial OXPHOS. By intracellular diffusion, oxygen reaches the inner membrane of mitochondria to provide electrons for inorganic phosphate and ADP is combined to give ATP. However, a study found that colorectal cells can respire under environment of 1% oxygen by diminished oxidative phosphorylation [56]. Yeast and higher eukaryotes have developed mechanisms to cope and survive under low oxygen conditions on both cellular and physiological level. Cells sense oxygen and induce a set of genes depending on the oxygen tension (pO_2) in the cell. Under hypoxia, cells express a different set of genes to cope with it. This hypoxic induction involves mainly HIF transcription factors.

HIFs are widely studied as they play a major role in cancer. HIFs are basic helix-loop-helix–PER–ARNT–SIM (bHLH–PAS) proteins, heterodimer of an α and a β subunit. The β subunit is stable whereas the stability of α subunit is linked to oxygen levels [57]. The human HIF family comprises HIF-1α, HIF-2α/EPAS, HIF-3α, ARNT (HIF-1β or bHLHe2) and ARNT2 (bHLHe1) [58]. Under hypoxia, HIF-1α stabilizes and heterodimerizes with HIF-1β. This complex translocates to the nucleus and binds to the motif A/(G)CGTG, known as hypoxic response elements (HREs) that are present in the promoters of the target genes of HIF-1 [59, 60]. Under normoxia, 2-oxoglutarate (2OG)-dependent dioxygenase enzymes called Prolyl

Hydroxylases (PHDs) hydroxylate HIF-1α at proline residues 402 and 564 [59]. This modification leads to their proteasomal degradation mediated by von Hippel–Lindau tumor suppressor protein (pVHL). HIF-1α is an 826 amino acids protein. The domain responsible for the oxygen-dependent stability of HIF-1α is oxygen-dependent degradation (ODD) domain that lies between amino acids 401 and 603 [61]. The absence of the C-terminal half of HIF-1α is shown to result in the loss of hypoxia-induced transactivation. It was later found that there are two hypoxia-inducible transactivation domains at the C-terminal of HIF-1α, TAD-N (aa 531–575) that lies within ODD and TAD-C (amino acids 786–826) [62, 63]. HIF-1 transcriptional activity is also repressed by FIH-1 (factor inhibiting HIF-1). FIH-1 binds to VHL and represses HIF-1 by recruiting histone deacetylases [64], which prevents its interaction with co-activators p300 and CREB binding protein (CBP). HIF-1 also acts on JmjC domain containing family of histone demethylases to induce transriptional changes under hypoxia.

1.7 JmjC domain containing histone demethylases induce chromatin modifications under hypoxia

To survive hypoxic conditions, cells undergo transcriptional changes. Under hypoxia, certain histone methylation marks are shown to be increased [65] (Fig. 1.5). Jumonji C (JmjC) domain containing proteins are found to be involved in the transcriptional changes under hypoxia, forming the missing connection between chromatin modifications and hypoxia response. JmjC domain containing family of proteins are mainly histone demethylases. Over 30 proteins have been identified in human and have been classified into seven groups of subfamilies - JHDM1, PHF2/PHF8, JARID, JHDM3/JMJD2, UTX/UTY, JHDM2, and JmjC domain only [66, 67]. Of all the JmjC family proteins, 24 proteins function as histone demethylases [68]. The

other family of histone demethylases are LSD1 family of Flavin-dependent monoamine oxidases (KDM1). In yeast Saccharomyces *cerevisiae,* five JmjC domain containing demethylases have been identified – Gis1, Rph1, Jhd1, Jhd2 and Ecm5. Rph1 and Jhd1 demethylate specifically H3K36, whereas Jhd2 demethylates H3K4me3 and H3K4me2 [69]. Ecm5 lacks cofactor binding residues in its JmjC domain, and Gis1 has been shown to have a weak demethylase activity towards H3. JmjC-KDMs belong to the 2-oxoglutarate (2OG) dependent oxygenase family and require molecular oxygen for their catalytic activity. Due to this requirement, histone demethylases can act as oxygen sensors. The following evidence support this notion. JMJD2 gene family was first identified and characterized by Katoh *et. al.* [70]. ChIP-chip and mRNA expression profiling on a genome-wide scale by Xia *et. al.* [71] showed that JmjC-containing histone demethylases are direct targets of HIF-1α – JMJD1A/KDM3A, JMJD2B/KDM4B, JMJD2C/KDM4C, JARID1C/KDM5C, JMJD3/KDM6B and JMJD6 [65, 72, 73]. In human liver cancer cell line HepG2 expression of 17 out of 22 proteins of JmjC family increased under hypoxia. In primary renal proximal epithelial cells, JMJD1A and JMJD2B were upregulated under hypoxia [74]. Hypoxia induced histone modifications observed in a hepatoma-derived cell line Hepa 1–6 showed an increase in H3K4me2 (2-fold), H3K4me3 (3.6-fold), H3K79me2 (1.4-fold), and a loss of H3K9ac [75]. A recent study by Hancock *et. al.* [76] showed that the activity of JMJD2A/KDM4A is highly sensitive to changes in oxygen concentration. Demethylation of H3K9me3, substrate of KDM4A, gradually decreases with decreasing concentration of oxygen.

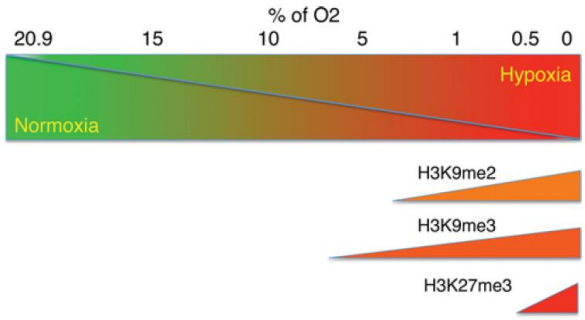


Figure 1.5. Hypoxia induces the increase of certain histone methylation marks. Diagram depicting the relationship between oxygen concentration and increased levels of histone methylation marks observed in several studies. Modified from Shmakova *et. al.* [65].

Another study by Park *et. al.* [77] showed that JMJD1A plays an important role in hypoxia-induced hepatocellular carcinoma cell growth. Under hypoxia, there is an increased occupancy of JMJD1A/KDM3A on the ADM gene promoter in human hepatocellular carcinoma cell lines HepG2 and Hep3B cells. The expression of JmjC domain containing lysyl hydroxylase, JMJD6/PTDSR in the human placenta is dependent on physiological oxygen levels [73]. Alahari *et. al.* showed that expression of JMJD6 is induced under low oxygen conditions and found that it is a downstream target of HIF-1. JMJD2C/KDM4C is shown to be highly expressed in human breast cancer cells by hypoxia in HIF-1-dependent manner. JMJD2C helps stimulate increased transcription of HIF-1 target genes by demethylating H3K9me3, which increases the binding of HIF-1 to the HREs of its target genes. In mice, knockdown of JMJD2C inhibits breast tumor growth [72]. KDM2A/JHDM1A is induced under hypoxia in a HIF-dependent manner [78]. KDM2A mRNA and protein increases under hypoxia through HIF-1. HIF-1α and HIF-1β are present at the KDM2A promoter and recruits RNA polymerase II under hypoxia. These results substantiate that the JmjC family of histone demethylases play an important role in the transcriptional response to hypoxia by chromatin modifications.

Recent studies in our lab have identified a novel heme-regulated protein in yeast-- JmjC domain containing transcription factor Gis1. Previously, Gis1 was known to be activated under nutrient stress. When the cells undergo post-diauxic shift (PDS), Gis1 binds to the PDS element T(T/A)AG_3AT on the promoter of its downstream genes mediating transcriptional response to nutrient stress [79]. Gis1 protein is 894 amino acids long and has several domains - Jumonji N (JmjN), Jumonji C (JmjC), a coiled-coil domain, two transcription activation domains (TAD), and two DNA binding C2H2 type zinc finger (ZnF) domains. It also has two CP or heme regulatory motifs, one in its JmjC domain and the other in its second zinc finger domain. Gis1 was identified in our lab as one of the fast responders to hypoxia and reoxygenation [53]. Since the effect of oxygen is largely mediated by heme, and histone demethylases are potential oxygen sensors, we studied if the transcriptional activity of Gis1 is regulated by heme (see Chapter 3).

References

1. Rao, A.U., et al., *Lack of heme synthesis in a free-living eukaryote.* Proc Natl Acad Sci U S A, 2005. **102**(12): p. 4270-5.

2. Heinemann, I.U., M. Jahn, and D. Jahn, *The biochemistry of heme biosynthesis.* Arch Biochem Biophys, 2008. **474**(2): p. 238-51.

3. Ferreira, G.C. and J. Gong, *5-Aminolevulinate synthase and the first step of heme biosynthesis.* J Bioenerg Biomembr, 1995. **27**(2): p. 151-9.

4. Ajioka, R.S., J.D. Phillips, and J.P. Kushner, *Biosynthesis of heme in mammals.* Biochim Biophys Acta, 2006. **1763**(7): p. 723-36.

5. Kumar, S. and U. Bandyopadhyay, *Free heme toxicity and its detoxification systems in human.* Toxicol Lett, 2005. **157**(3): p. 175-88.

6. Wagener, F.A., et al., *Different faces of the heme-heme oxygenase system in inflammation.* Pharmacol Rev, 2003. **55**(3): p. 551-71.

7. Khan, A.A. and J.G. Quigley, *Control of intracellular heme levels: heme transporters and heme oxygenases.* Biochim Biophys Acta, 2011. **1813**(5): p. 668-82.

8. Poulos, T.L., *Heme enzyme structure and function.* Chem Rev, 2014. **114**(7): p. 3919-62.

9. Immenschuh, S., et al., *Heme as a Target for Therapeutic Interventions.* Front Pharmacol, 2017. **8**: p. 146.

10. Tolosano, E. and F. Altruda, *Hemopexin: structure, function, and regulation.* DNA Cell Biol, 2002. **21**(4): p. 297-306.

11. Wu, N., et al., *Negative feedback maintenance of heme homeostasis by its receptor, Rev-erbalpha.* Genes Dev, 2009. **23**(18): p. 2201-9.

12. Bock, K.W., F. De Matteis, and W.N. Aldridge, *Heme and Hemoproteins*. 1978, New York: Springer-Verlag.

13. Padmanaban, G., V. Venkateswar, and P.N. Rangarajan, *Haem as a multifunctional regulator.* Trends Biochem Sci, 1989. **14**(12): p. 492-6.

14. Chen, J.J. and I.M. London, *Regulation of protein synthesis by heme-regulated eIF-2 alpha kinase.* Trends Biochem Sci, 1995. **20**(3): p. 105-8.

15. Zhang, L., *HEME BIOLOGY: The Secret Life of Heme in Regulating Diverse Biological Processes*. 2011: World Scientific.

16. Hooda, J., A. Shah, and L. Zhang, *Heme, an essential nutrient from dietary proteins, critically impacts diverse physiological and pathological processes.* Nutrients, 2014. **6**(3): p. 1080-102.

17. Anderson, K.E., et al., *Disorders of heme biosynthesis: X-linked sideroblastic anemia and the porphyrias*, in *The metabolic and molecular bases of inherited disease*, C.R. Scriver, et al., Editors. 2001, The McGraw-Hill Companies, Inc.: New York. p. 2991-3062.

18. Sassa, S., *Novel effects of heme and heme-related compounds in biological systems.* Current Med. Chem., 1996. **3**: p. 273-290.

19. Suzuki, H., et al., *Heme regulates gene expression by triggering Crm1-dependent nuclear export of Bach1*. Embo J, 2004. **23**(13): p. 2544-53.

20. Zenke-Kawasaki, Y., et al., *Heme induces ubiquitination and degradation of the transcription factor Bach1*. Mol Cell Biol, 2007. **27**(19): p. 6962-71.

21. Hira, S., et al., *Bach1, a heme-dependent transcription factor, reveals presence of multiple heme binding sites with distinct coordination structure.* IUBMB Life, 2007. **59**(8-9): p. 542-51.

22. Uma, S., et al., *The N-terminal region of the heme-regulated eIF2alpha kinase is an autonomous heme binding domain.* Eur J Biochem, 2000. **267**(2): p. 498-506.

23. Rafie-Kolpin, M., et al., *Two heme-binding domains of heme-regulated eukaryotic initiation factor-2alpha kinase. N terminus and kinase insertion.* J Biol Chem, 2000. **275**(7): p. 5171-8.

24. Sengupta, A., T. Hon, and L. Zhang, *Heme deficiency suppresses the expression of key neuronal genes and causes neuronal cell death.* Brain Res Mol Brain Res, 2005. **137**(1-2): p. 23-30.

25. Zhu, Y., et al., *Heme deficiency interferes with the Ras-mitogen-activated protein kinase signaling pathway and expression of a subset of neuronal genes.* Cell Growth Differ, 2002. **13**(9): p. 431-9.

26. Chernova, T., P. Nicotera, and A.G. Smith, *Heme deficiency is associated with senescence and causes suppression of N-methyl-D-aspartate receptor subunits expression in primary cortical neurons.* Mol Pharmacol, 2006. **69**(3): p. 697-705.

27. Chernova, T., et al., *Neurite degeneration induced by heme deficiency mediated via inhibition of NMDA receptor-dependent extracellular signal-regulated kinase 1/2 activation.* J Neurosci, 2007. **27**(32): p. 8475-85.

28. Raghuram, S., et al., *Identification of heme as the ligand for the orphan nuclear receptors REV-ERBalpha and REV-ERBbeta.* Nat Struct Mol Biol, 2007. **14**(12): p. 1207-13.

29. Yin, L., et al., *Rev-erbalpha, a heme sensor that coordinates metabolic and circadian pathways.* Science, 2007. **318**(5857): p. 1786-9.

30. Faller, M., et al., *Heme is involved in microRNA processing.* Nat Struct Mol Biol, 2007. **14**(1): p. 23-9.

31. Tang, X.D., et al., *Haem can bind to and inhibit mammalian calcium-dependent Slo1 BK channels.* Nature, 2003. **425**(6957): p. 531-5.

32. Wang, S., S. Publicover, and Y. Gu, *An oxygen-sensitive mechanism in regulation of epithelial sodium channel.* Proc Natl Acad Sci U S A, 2009. **106**(8): p. 2957-62.

33. Sahoo, N., et al., *Heme impairs the ball-and-chain inactivation of potassium channels.* Proc Natl Acad Sci U S A, 2013. **110**(42): p. E4036-44.

34. Kim, H.J., et al., *Structure, function, and assembly of heme centers in mitochondrial respiratory complexes.* Biochim Biophys Acta, 2012. **1823**(9): p. 1604-16.

35. Wallace, D.C., W. Fan, and V. Procaccio, *Mitochondrial energetics and therapeutics.* Annu Rev Pathol, 2010. **5**: p. 297-348.

36. Efremov, R.G., R. Baradaran, and L.A. Sazanov, *The architecture of respiratory complex I.* Nature, 2010. **465**(7297): p. 441-5.

37. Efremov, R.G. and L.A. Sazanov, *Structure of the membrane domain of respiratory complex I.* Nature, 2011. **476**(7361): p. 414-20.

38. Bunn, H.F. and R.O. Poyton, *Oxygen sensing and molecular adaptation to hypoxia.* Physiol Rev, 1996. **76**(3): p. 839-85.

39. Paoli, M., J. Marles-Wright, and A. Smith, *Structure-function relationships in heme-proteins.* DNA Cell Biol, 2002. **21**(4): p. 271-80.

40. Zhang, L. and L. Guarente, *HAP1 is nuclear but is bound to a cellular factor in the absence of heme.* J Biol Chem, 1994. **269**(20): p. 14643-7.

41. Mense, S.M. and L. Zhang, *Heme: a versatile signaling molecule controlling the activities of diverse regulators ranging from transcription factors to MAP kinases.* Cell Res, 2006. **16**(8): p. 681-92.

42. Alam, M.Z., S. Devalaraja, and M. Haldar, *The Heme Connection: Linking Erythrocytes and Macrophage Biology*. Front Immunol, 2017. **8**: p. 33.

43. Hon, T., et al., *The heme activator protein Hap1 represses transcription by a heme-independent mechanism in Saccharomyces cerevisiae.* Genetics, 2005. **169**(3): p. 1343-52.

44. Vignali, M., et al., *ATP-dependent chromatin-remodeling complexes.* Mol Cell Biol, 2000. **20**(6): p. 1899-910.

45. Clapier, C.R. and B.R. Cairns, *The biology of chromatin remodeling complexes.* Annu Rev Biochem, 2009. **78**: p. 273-304.

46. Wang, W., et al., *Diversity and specialization of mammalian SWI/SNF complexes.* Genes Dev, 1996. **10**(17): p. 2117-30.

47. Sudarsanam, P. and F. Winston, *The Swi/Snf family nucleosome-remodeling complexes and transcriptional control.* Trends Genet, 2000. **16**(8): p. 345-51.

48. Kodaki, T., et al., *The SNF2/SWI2/GAM1/TYE3/RIC1 gene is involved in the coordinate regulation of phospholipid synthesis in Saccharomyces cerevisiae.* J Biochem, 1995. **117**(2): p. 362-8.

49. Peterson, C.L. and I. Herskowitz, *Characterization of the yeast SWI1, SWI2, and SWI3 genes, which encode a global activator of transcription.* Cell, 1992. **68**(3): p. 573-83.

50. Shain, A.H. and J.R. Pollack, *The spectrum of SWI/SNF mutations, ubiquitous in human cancers.* PLoS One, 2013. **8**(1): p. e55119.

51. Halliday, G.M., et al., *SWI/SNF: a chromatin-remodelling complex with a role in carcinogenesis.* Int J Biochem Cell Biol, 2009. **41**(4): p. 725-8.

52. Euskirchen, G.M., et al., *Diverse roles and interactions of the SWI/SNF chromatin remodeling complex revealed using global approaches.* PLoS Genet, 2011. **7**(3): p. e1002008.

53. Henke, R.M., et al., *Hypoxia elicits broad and systematic changes in protein subcellular localization.* Am J Physiol Cell Physiol, 2011. **301**(4): p. C913-28.

54. Dastidar, R.G., et al., *The nuclear localization of SWI/SNF proteins is subjected to oxygen regulation.* Cell Biosci, 2012. **2**(1): p. 30.

55. Kenneth, N.S., et al., *SWI/SNF regulates the cellular response to hypoxia.* J Biol Chem, 2009. **284**(7): p. 4123-31.

56. Frezza, C., P.J. Pollard, and E. Gottlieb, *Inborn and acquired metabolic defects in cancer.* J Mol Med (Berl), 2011. **89**(3): p. 213-20.

57. Kaelin, W.G., Jr., *Cancer and altered metabolism: potential importance of hypoxia-inducible factor and 2-oxoglutarate-dependent dioxygenases.* Cold Spring Harb Symp Quant Biol, 2011. **76**: p. 335-45.

58. Graham, A.M. and J.S. Presnell, *Hypoxia Inducible Factor (HIF) transcription factor family expansion, diversification, divergence and selection in eukaryotes.* PLoS One, 2017. **12**(6): p. e0179545.

59. Johnson, A.B. and M.C. Barton, *Hypoxia-induced and stress-specific changes in chromatin structure and function.* Mutat Res, 2007. **618**(1-2): p. 149-62.

60. Wenger, R.H., *Mammalian oxygen sensing, signalling and gene regulation.* J Exp Biol, 2000. **203**(Pt 8): p. 1253-63.

61. Huang, L.E., et al., *Regulation of hypoxia-inducible factor 1alpha is mediated by an O2-dependent degradation domain via the ubiquitin-proteasome pathway.* Proc Natl Acad Sci U S A, 1998. **95**(14): p. 7987-92.

62. Jiang, B.H., et al., *Transactivation and inhibitory domains of hypoxia-inducible factor 1alpha. Modulation of transcriptional activity by oxygen tension.* J Biol Chem, 1997. **272**(31): p. 19253-60.

63. Lando, D., et al., *FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor.* Genes Dev, 2002. **16**(12): p. 1466-71.

64. Mahon, P.C., K. Hirota, and G.L. Semenza, *FIH-1: a novel protein that interacts with HIF-1alpha and VHL to mediate repression of HIF-1 transcriptional activity.* Genes Dev, 2001. **15**(20): p. 2675-86.

65. Shmakova, A., et al., *Chromatin and oxygen sensing in the context of JmjC histone demethylases.* Biochem J, 2014. **462**(3): p. 385-95.

66. Blair, L.P., et al., *Epigenetic Regulation by Lysine Demethylase 5 (KDM5) Enzymes in Cancer*. Cancers (Basel), 2011. **3**(1): p. 1383-404.

67. Klose, R.J., E.M. Kallin, and Y. Zhang, *JmjC-domain-containing proteins and histone demethylation.* Nat Rev Genet, 2006. **7**(9): p. 715-27.

68. Hojfeldt, J.W., K. Agger, and K. Helin, *Histone lysine demethylases as targets for anticancer therapy.* Nat Rev Drug Discov, 2013. **12**(12): p. 917-30.

69. Kwon, D.W. and S.H. Ahn, *Role of yeast JmjC-domain containing histone demethylases in actively transcribed regions.* Biochem Biophys Res Commun, 2011. **410**(3): p. 614-9.

70. Katoh, M. and M. Katoh, *Identification and characterization of JMJD2 family genes in silico.* Int J Oncol, 2004. **24**(6): p. 1623-8.

71. Xia, X., et al., *Integrative analysis of HIF binding and transactivation reveals its role in maintaining histone methylation homeostasis.* Proc Natl Acad Sci U S A, 2009. **106**(11): p. 4260-5.

72. Luo, W., et al., *Histone demethylase JMJD2C is a coactivator for hypoxia-inducible factor 1 that is required for breast cancer progression.* Proc Natl Acad Sci U S A, 2012. **109**(49): p. E3367-76.

73. Alahari, S., M. Post, and I. Caniggia, *Jumonji Domain Containing Protein 6: A Novel Oxygen Sensor in the Human Placenta.* Endocrinology, 2015. **156**(8): p. 3012-25.

74. Beyer, S., et al., *The histone demethylases JMJD1A and JMJD2B are transcriptional targets of hypoxia-inducible factor HIF*. J Biol Chem, 2008. **283**(52): p. 36542-52.

75. Johnson, A.B., N. Denko, and M.C. Barton, *Hypoxia induces a novel signature of chromatin modifications and global repression of transcription.* Mutat Res, 2008. **640**(1-2): p. 174-9.

76. Hancock, R.L., et al., *The Activity of JmjC Histone Lysine Demethylase KDM4A is Highly Sensitive to Oxygen Concentrations.* ACS Chem Biol, 2017. **12**(4): p. 1011-1019.

77. Park, S.J., et al., *The histone demethylase JMJD1A regulates adrenomedullin-mediated cell proliferation in hepatocellular carcinoma under hypoxia.* Biochem Biophys Res Commun, 2013. **434**(4): p. 722-7.

78. Batie, M., et al., *KDM2 Family Members are Regulated by HIF-1 in Hypoxia.* Cells, 2017. **6**(1).

79. Pedruzzi, I., et al., *Saccharomyces cerevisiae Ras/cAMP pathway controls post-diauxic shift element-dependent transcription through the zinc finger protein Gis1*. EMBO J, 2000. **19**(11): p. 2569-79.

CHAPTER 2

THE SWI3 PROTEIN PLAYS A UNIQUE ROLE IN REGULATING AEROBIC RESPIRATION IN EUKARYOTES

Authors - Sneha Lal, Md Maksudul Alam, Jagmohan Hooda, Ajit Shah, Thai M Cao, Zhenyu Xuan and Li Zhang

Department of Biological Sciences

Center for Systems Biology

The University of Texas at Dallas

800 West Campbell Road

Richardson, Texas 75080-3021

Running head: Swi3 as a regulator of cellular bioenergetics

Reprint from Bioscience Reports, 2016 (Open Access)

- 36 (3) e00350; https://doi.org/10.1042/BSR20160083

ABSTRACT

Recent experimental evidence increasingly shows that the dysregulation of cellular bioenergetics is associated with a wide array of common human diseases, including cancer, neurological diseases, and diabetes. Aerobic respiration provides a vital source of cellular energy for most eukaryotic cells, particularly high energy demanding cells. Although the metabolic pathways and enzymes involved in aerobic respiration and oxidative phosphorylation are well characterized, the understanding of how aerobic respiration is globally regulated is very limited. In this report, we performed an array of biochemical and genetic experiments and computational analysis to directly evaluate the function of Swi3 and its human homologues in regulating aerobic respiration. First, we showed, by computational analysis and measurements of oxygen consumption rates and promoter activities, that Swi3, not Swi2, regulates genes encoding functions involved in aerobic respiration and oxygen consumption. Biochemical analysis showed that the levels of mitochondrial oxidative phosphorylation complexes are substantially increased in *Δswi3* cells, compared to the parent cells. Additionally, our data showed that Swi3 strongly impacts heme/oxygen-dependent activation of aerobic respiration gene promoters while Swi2 impacts only the basal, heme-independent activities of these promoters. We found that oxygen consumption and growth rates increase with increased expression of aerobic respiration genes in *Δswi3* cells in air. Furthermore, using computational analysis, we showed that the mammalian Swi3 BAF155 and BAF170 regulate aerobic respiration in HeLa cells. Together, these experimental and computational data showed that Swi3 and its mammalian homologues are key regulators in regulating aerobic respiration.

2.1 Introduction

Components of the SWI/SNF chromatin remodeling complex play essential roles in the development and proper functioning of a wide array of tissues and cell types, ranging from stem cells to neural and skin cells [1-4]. Their dysfunction is implicated in many cancers and neurological disorders. It is well known that Swi3 and its human homologues BAF155 and BAF170 are essential components of the SWI/SNF complexes, with Swi2 providing ATPase activity for chromatin remodeling [5-11]. The SWI/SNF complexes are found in virtually all eukaryotes [12]. Purified SWI/SNF complexes contain 10-12 polypeptides and have an apparent molecular mass of 1.14 MDa in yeast and about 2 MDa in mammals [5, 6]. As part of this complex, Swi3 controls SWI/SNF assembly, ATP-dependent H2A-H2B displacement, as well as recruitment to target genes [7, 8]. This complex acts at an array of promoters to promote chromatin remodeling and transcriptional activation [13, 14]. The target genes of SWI/SNF components constitute more than 10% of all yeast genes [15]. It is worth noting that both genetic data from yeast studies and ChIP-Seq data from mammalian cells showed that the target genes of SWI/SNF components do not completely overlap. Particularly Swi3 and its mammalian homologues BAF155 and BAF170 can target a good number of genes and genomic locations in the absence of Swi2 and other components of the SWI/SNF complex [15, 16], suggesting that they may have unique functions in gene regulation.

Furthermore, several lines of previous experimental evidence suggest that SWI/SNF proteins have important functions in oxygen regulation of gene expression. Firstly, by using fluorescent live cell imaging, we showed that six components of the SWI/SNF complex--Swi3, Snf5, Snf6, Snf11, Snf12 and Swp82--require oxygen for nuclear localization [17, 18]. Under

hypoxia, these proteins accumulate in the cytosol; upon reoxygenation, they relocalize to the nucleus. Notably, the changes in protein localization in response to hypoxia or reoxygenation precede the changes in transcriptome, showing a causal role of protein relocalization in promoting transcriptional changes [17]. Further, we characterized the time courses of relocalization of hypoxia-altered nuclear proteins in response to hypoxia and reoxygenation [18]. We found that Swi3, as well as 16 other nuclear proteins, responds to both hypoxia and reoxygenation in shorter times than the rest of the hypoxia-altered proteins. Secondly, we analyzed mRNAs and proteins whose levels are regulated by oxygen levels and found that many oxygen-regulated genes are targets of Swi3 and Swi2 [19]. Thirdly, Gat-Viks and colleagues performed a gene sequence variation study identifying regulatory-linkage modules based on DNA sequence polymorphism and expression data [20]. They showed that Swi3 is a dominant regulator in the control of respiratory gene expression: the effect of swi3 deletion is stronger than that of known respiratory regulators, including Hap2/3/4/5, Mot3 and Rox1 [20]. All these results point out that Swi3 likely plays a unique role in oxygen regulation and the regulation of aerobic respiration.

Therefore, we decided to further ascertain the function of Swi3 in oxygen regulation and aerobic respiration. In this report, we confirmed that Swi3, but not Swi2, has a novel function in suppressing aerobic respiration and oxygen consumption. It is worth noting that this Swi3 function in suppressing aerobic respiration genes is distinct from its function in the SWI/SNF complex, which promotes chromatin remodeling and enhances transcription [13, 14]. We showed that in cells with the SWI3 gene deleted, the expression of genes encoding mitochondrial oxidative phosphorylation complexes is upregulated. Additionally, we performed a

computational analysis of genes bound by SWI/SNF proteins in mammalian cells, identified by the Snyder lab [16]. We found that the Swi3 homologues BAF155 and BAF170, but not the Swi2 homologue Brg1, are preferentially associated with genes encoding oxidative phosphorylation functions. Together, these results uncover a unique role of Swi3 and its mammalian homologues in aerobic respiration and cellular bioenergetics.

2.2 Materials and Methods

2.2.1 Yeast strains and plasmids

The yeast knockout and parent BY4741 (*MATa his3Δ1 leu2Δ 0 met15Δ0 ura3Δ0*) strains were purchased from Open Biosystems, Inc. The *HEM1* gene in the BY4741*Δhem1*, *Δswi3Δhem1*, *Δswi2Δhem1* strains were deleted as described previously [1]. To generate the BY4741*Δswi2Δswi3* strain, the BY4741 *Δswi3* strain was transformed with PCR products containing the *LEU2* gene in the middle and 44 bps of sequences flanking the ORF sequence of *SWI2* on each end. The knockout strains were confirmed by using PCR analysis of the corresponding genomic DNA. Sequences for PCR primers are available upon request. The *CYC1-lacZ* and *CYC7-lacZ* reporters were described previously [2, 3]. The *CYC1-lacZ* reporter contains -312 to -1 of the *CYC1* promoter region, while the *CYC7-lacZ* reporter contains -700 to -1 of the *CYC7* promoter region.

2.2.2 Cell growth and β-galactosidase assays

Yeast cells were grown in rich YPD or synthetic complete media, as described previously [4, 5]. Cell density was determined by measuring optical density at 600 nm. To determine β-galactosidase levels from reporter genes in *Δhem1* cells bearing the *CYC1-lacZ* or *CYC7-lacZ* reporter, cells were grown in synthetic complete medium containing a limiting amount of the

heme precursor 5-aminolevulinate (2.5 μg/ml) or a high amount of 5-aminolevulinate (250 μg/ml). Note that *CYC1* and *CYC7* are not subjected to glucose suppression [3, 6-8]. Cells were collected after they reached an optical density (600 nm) of approximately 1.0-1.5. Collected cells were then subjected to chloroform permeabilization β-galactosidase assays (in Miller units), as described previously [9].

2.2.3 Measurement of oxygen consumption rate

Oxygen consumption was measured, as described previously [10]. HeLa cells were cultured to about 80% confluency, and were then trypsinized and resuspended in fresh, air-saturated medium. For each measurement, 10^6 cells (in 350 μl) were introduced in the chamber of an Oxygraph system (Hansatech Instruments), with a Clark-type electrode placed at the bottom of the respiratory chamber. During measurements, the chamber was thermostated at 37 ^oC by a circulating water bath. An electromagnetic stirrer bar was used to mix the contents of the chamber. For measuring oxygen consumption rate in yeast cells, 10^8 cells (in 500 μl) were used, and the temperature was kept at 25 ^oC.

2.2.4 Analysis of mitochondrial oxidative phosphorylation complexes

Yeast parent BY4741, *Δswi2* and *Δswi3* deletion strains were grown in medium containing glucose or lactate (Lac) (note that *Δswi3* cells cannot grow in lactate medium) to mid-log phase. Cells were collected, and mitochondria were purified as described [11]. Purified mitochondria were solubilized and analyzed on 4-12% acrylamide gradient Blue native PAGE gels, as described [12, 13]. 150 μg of total mitochondrial proteins was loaded in each lane.

2.2.5 Computational analysis of Swi3, BAF155, and BAF 170 target genes

The data for yeast Swi2 and Swi3 target genes were taken from Hu et al., 2007 [14]. Those genes identified as targets of Swi2 and Swi3 were used to perform the functional category enrichment analysis with DAVID (http://david.abcc.ncifcrf.gov/). All the KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways and the GO (gene ontology) terms from biological processes (BP) and molecular functions (MF) were analyzed. The ChIP-Seq data for BAF155, BAF170 and Brg1 targets are taken from Euskirchen et al., 2011 [15]. To identify the target genes of these proteins, we first identified genes whose promoters (defined as +/-2.5Kb of the transcription start site) are located in regions bound by these proteins. Then, we performed GO and KEGG pathway analysis by using the NIH DAVID program. The most significant GO groups and pathways with p values less than 0.05 were compared.

2.3 Results

2.3.1 Computational analysis shows that Swi3 targets, but not Swi2 targets, are enriched in genes encoding functions involved in oxidation and reduction

Previously, Hu et al. used gene expression data and directed-weighted graph modeling and regulatory epistasis analysis to characterize the regulatory network involving 263 transcription factors and to identify their target genes [14]. These transcription factors include several components of the SWI/SNF complexes. Specifically, they identified 426 target genes for Swi2, with 63 unique to Swi2. Additionally, they identified 391 targets for Swi3, with 82 unique to Swi3. We performed GO and KEGG analyses of these unique targets and found that their function categories are completely different. While Swi3 targets are enriched in the enzymes involved in oxidation and reduction, Swi2 targets are enriched in those involved in ribosome

biogenesis and translation (see Table 2.1 for examples). No statistically significant GO groups of the Swi2 targets are relevant to oxidation and reduction, whereas no statistically significant GO groups of the Swi3 targets are relevant to ribosome (not shown). This analysis provides further support for investigating the unique function of Swi3 in regulating oxygen metabolism.

2.3.2 Swi3, but not Swi2, strongly impacts oxygen consumption and aerobic respiration

To investigate the function of Swi3 and other SWI/SNF components in aerobic respiration, we measured the rates of oxygen consumption in various deletion strains and compared them with that of the parent strain BY4741 (Fig. 2.1). Evidently, the rate of oxygen consumption was significantly elevated in *Δswi3* cells, compared to the parent BY4741 cells. However, the rate of oxygen consumption was not enhanced in *Δswi2* cells or the cells with one of the other SWI/SNF genes deleted, except for *Δtaf14* cells (Fig. 2.1).

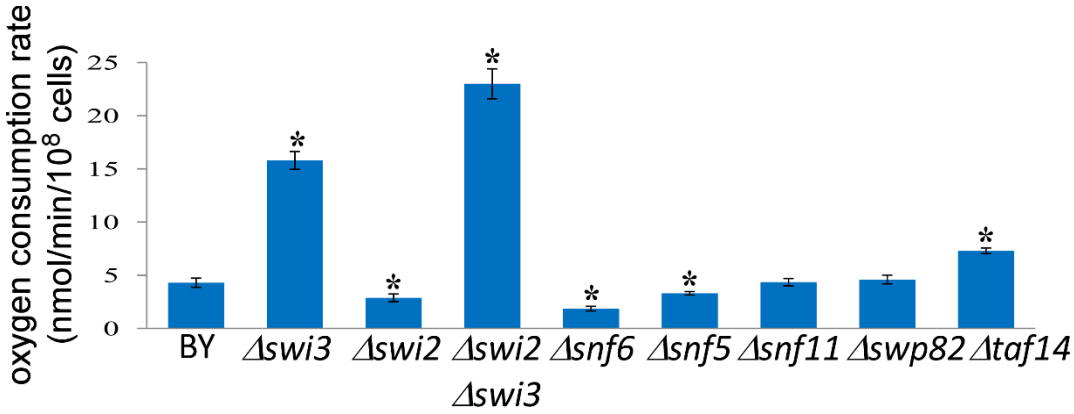


Figure 2.1. The effect of deleting the genes encoding components of the SWI/SNF complex on oxygen consumption rate. Yeast parent BY4741 (BY) and knockout strains were grown to mid-log phase and collected for measuring oxygen consumption. The data shown are averages of at least three independent measurements. For statistical analysis, the values were compared to those in the parent strain (BY), by using Welch 2-sample t-test. *, p value < 0.005.

Notably, deletion of the SWI2 gene in *Δswi3* cells did not diminish intensified oxygen consumption, suggesting that Swi3 acts independently of Swi2. Further, we measured the levels

of mitochondrial oxidative phosphorylation complexes in the parent and *Δswi3* cells. We prepared purified mitochondria and analyzed them by blue native (BN) gel electrophoresis [13, 16] (Fig. 2.2A). We found that increased oxygen consumption in *Δswi3* cells is correlated with increased levels of mitochondrial oxidative phosphorylation complexes. As expected, the levels of the complexes isolated from the parent cells grown in lactate (see V_{Dimer}, V, and III_{Dimer}, Fig. 2.2A, BY lac) were much higher than those from the parent (BY, Fig. 2.2A) or *Δswi2* cells grown in glucose.

Notably, the level of the complexes in *Δswi3* cells grown in glucose was significantly higher than that in parent cells grown in glucose (BY). The level of the complexes was somewhat increased in *Δswi2* cells, but lower than that in *Δswi3* cells. The same samples were also analysed by using SDS/PAGE (Figure 2.2B), to show that the amounts of total proteins in these samples were similar. This result showing increased level of oxidative phosphorylation complexes is consistent with enhanced oxygen consumption, in *Δswi3* cells.

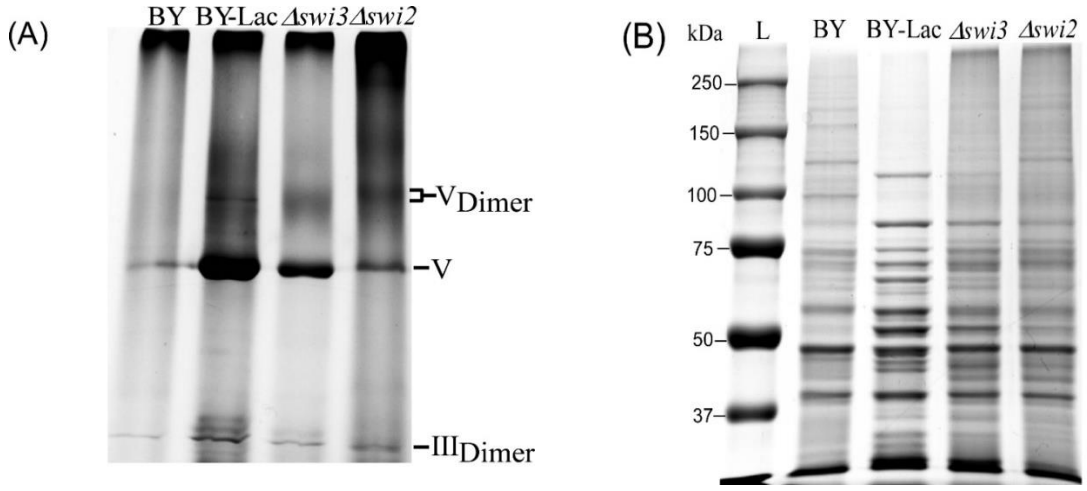


Figure 2.2. The effect of deleting the *SWI3* gene on the expression of mitochondrial oxidative phosphorylation complexes. Yeast parent BY4741 (BY), *Δswi2* and *Δswi3* deletion strains were

grown in medium containing glucose or lactate (Lac) (note that *Δswi2* and *Δswi3* cells cannot grow in lactate medium) to mid-log phase. Cells were collected, and mitochondria were purified. Purified mitochondria were solubilized and analyzed on 4-12% acrylamide gradient BN gels (A). In (B), the same samples were analysed by using SDS/PAGE.

2.3.3 Swi3, but not Swi2, is critical for heme-dependent activation of genes encoding functions involved in aerobic respiration

To further probe the mechanism by which Swi3 impacts aerobic respiration, we examined the effect of deleting *SWI2* or *SWI3* on the promoter activities of two previously characterized aerobic respiration genes *CYC1* and *CYC7*, encoding cytochrome c iso-1 and iso-2, respectively [2, 3, 17]. It is worth mentioning that the regulation of these genes, which are not subjected to glucose suppression [3, 6-8], are distinct from those that are activated by the SWI/SNF complex and are subjected to glucose suppression, such as *SUC2*, *GAL1*, and *GAL10* [18]. We measured the β-galactosidase activities of the full *CYC1* promoter-*lacZ* and *CYC7* promoter-*lacZ* reporters in *Δswi2* and *Δswi3* cells, under heme-sufficient and heme-deficient conditions (Fig. 2.3). Note that intracellular heme levels can be controlled by deleting the *HEM1* gene and adding back different levels of the heme precursor 5-aminolevulinic acid (ALA). Additionally, the level of heme synthesis is tightly controlled by oxygen level [4]. In heme-deficient *Δswi2* cells, the activities of the reporters were significantly higher than those in heme-deficient parent cells (Fig. 2.3). High heme levels in heme-sufficient *Δswi2* cells did not cause a further increase in reporter activities. This shows that Swi2 generally affects the promoter activities in a heme (respiration)-independent manner. In heme-deficient *Δswi3* cells, the activities of the reporters were higher than those in heme-deficient parent cells, but were not as high as those in heme-deficient *Δswi2* cells (Fig. 2.3).

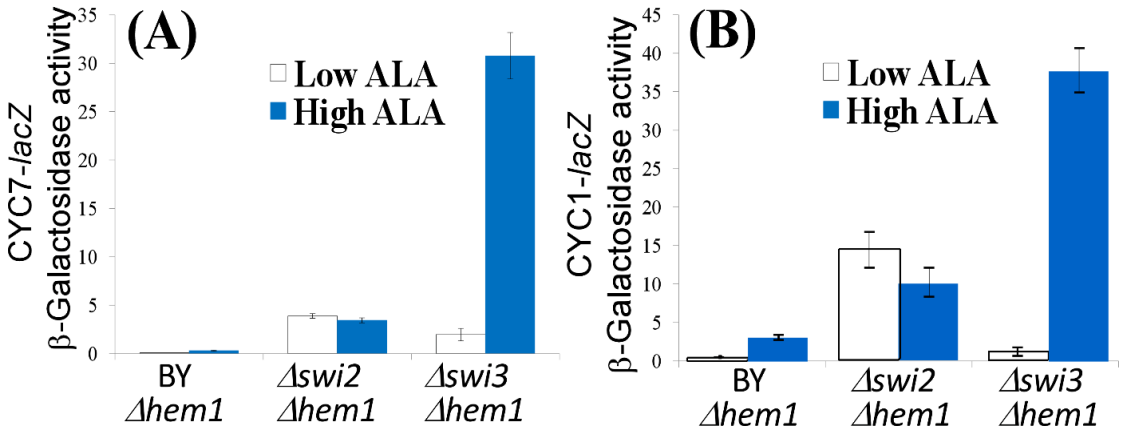


Figure 2.3. Swi2 and Swi3 exert differential effects on respiration gene transcription. Deletion of the SWI3 gene, not SWI2, causes a significant increase in the *CYC7* (A) and *CYC1* (B) promoter reporter activities in heme-sufficient cells. Yeast parent BY4741*Δhem1* (BY*Δhem1*), BY4741*Δswi2Δhem1* (*Δswi2Δhem1*) and BY4741*Δswi3Δhem1* (*Δswi3Δhem1*) strains bearing the *CYC1-lacZ* or *CYC7-lacZ* reporter plasmid were grown in the presence of 2.5 μg/ml (low ALA) or 250 μg/ml (high ALA) heme precursor 5-aminolevulinic acid (ALA). β-galactosidase activities were measured and plotted here. Data shown here are averages from at least three independent measurements.

Notably, high heme levels in heme-sufficient *Δswi3* cells further caused a substantial increase in reporter activities. This shows that Swi3 strongly impacts heme/oxygen-dependent promoter activities but exerts a weaker heme (respiration)-independent effect on the promoters than Swi2.

2.3.4 Increased activation of aerobic respiration gene expression correlates with intensified oxygen consumption and cell growth in *Δswi3* cells

To further investigate the association of heme and aerobic respiration gene expression with oxygen consumption in *Δswi3* cells, we measured the rates of oxygen consumption in the parent BY4741*Δhem1* and *Δswi3Δhem1* cells and compared them to *CYC7* promoter activities, under increasing concentrations of the heme precursor ALA. Fig. 2.4 shows that as heme levels

increased, the rate of oxygen consumption, along with the *CYC7* reporter activity, increased substantially in *Δswi3Δhem1* cells, but it did not increase in BY4741*Δhem1* cells.

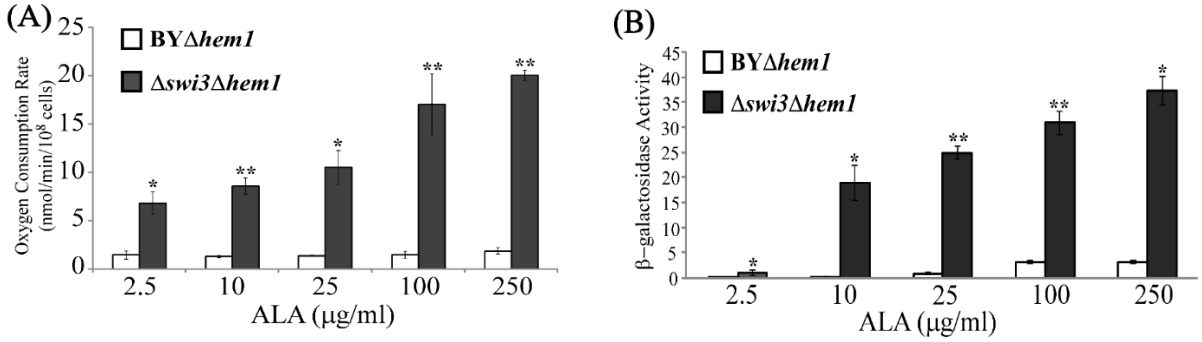


Figure 2.4. Heme promotes the rates of oxygen consumption (A) and CYC7 promoter activity (B) in *Δswi3* cells. Yeast parent BY4741*Δhem1* (BY*Δhem1*), and BY4741*Δswi3Δhem1* (*Δswi3Δhem1*) cells bearing the *CYC7-lacZ* reporter were grown in the presence of indicated levels of heme precursor ALA. Cells were grown to mid-log phase and collected for measuring oxygen consumption or β-galactosidase activities. The data shown are averages of at least three independent measurements. For statistical analysis, the values were compared to those in the parent strain (BY), by using Welch 2-sample t-test. *, p value < 0.005; **, p value < 0.001.

Further, we found that in air, the rate of *Δswi3* cell growth was increased compared to the parent cells (Fig. 2.5A). However, under hypoxia, in the absence of aerobic respiration, *Δswi3* cell growth rate was much lower than the parent cells (Fig. 2.5B). These results together show that the loss of Swi3 function promotes the expression of aerobic respiration genes and the formation of mitochondrial oxidative phosphorylation complexes, leading to increased oxygen consumption and cell growth in air. Additionally, the data suggest that the function of Swi3 in aerobic cells is distinct from its function in hypoxic cells.

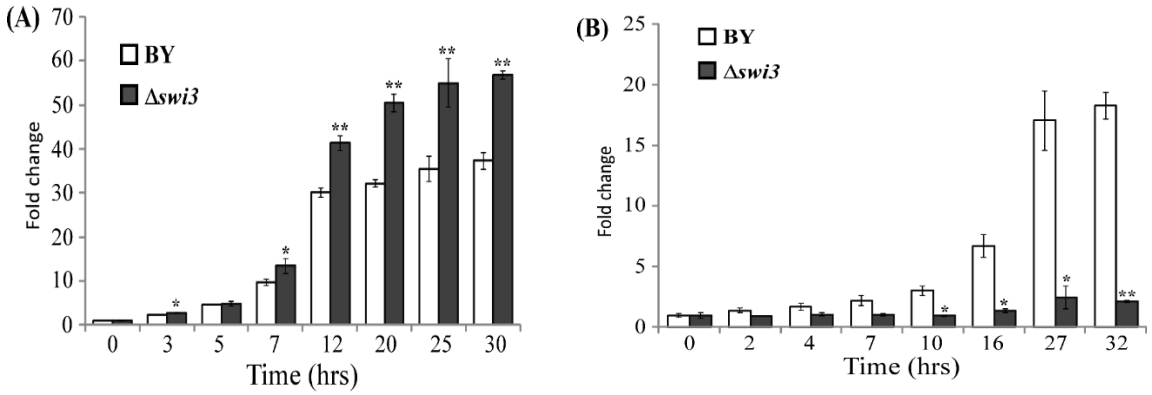


Figure 2.5. Deletion of the *SWI3* gene selectively enhances cell growth rate in air. Yeast parent BY4741 (BY) and BY4741*Δswi3* (*Δswi3*) were grown in air (A) or under hypoxia (B). Cells were grown to mid-log phase and collected for measuring optical density. Fold change was calculated by dividing the optical density at the indicated time points with that at the designated 0 hour time. The data shown are averages of at least three independent measurements. For statistical analysis, the values were compared to those in the parent strain (BY), by using Welch 2-sample t-test. *, p value < 0.005; **, p value < 0.001.

2.3.5 Computational analysis of genome-wide ChIP-Seq data about BAF proteins suggest that the human Swi3 homoloues BAF155 and BAF170 are important for aerobic respiration gene expression

The functions of SWI/SNF proteins are highly conserved from yeast to humans. Particularly, Swi3 has two homologues in humans, namely BAF155 and BAF170. These three proteins contain three conserved functional domains, namely SWIRM, SANT, and LZ domains [19, 20]. To comprehensively survey the genome-wide localization of SWI/SNF proteins, three ChIP-Seq studies have been performed in ES cells, T helper cells, and HeLa cells [15, 21, 22]. In particular, the Synder lab detected and compared the localization patterns of Brg1, BAF155, BAF170, and Ini1 [15]. Analysis of their data (see Table 2.2) showed that BAF155 and BAF170

localize to many regions/genes where Brg1 does not, suggesting that BAF155 and BAF170 have other functions besides those requiring Brg1.

To probe whether the role of Swi3 in aerobic respiration is conserved in mammalian cells, we analyzed and compared the regions bound by BAF155, BAF170 and Brg1, using ChIP-Seq data from Euskirchen and colleagues [15] (Table 2.2). First, we identified genes whose promoters (defined as +/-2.5Kb of the transcription start site) are located in regions bound by these proteins (Gene Promoters in Table 1). Then, we performed GO (gene ontology) and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis. Strikingly, the analysis (Table 2.2) showed that among the genes bound by both BAF155 and BAF170, but not by Brg1, a significant number [47] of them encode enzyme subunits of the mitochondrial oxidative phosphorylation complexes. In contrast, among the genes bound by BAF155, BAF170 and Brg1, those associated with oxidative phosphorylation were not present. No statistically significant GO groups of the Brg1 targets are relevant to oxidative phosphorylation. The results suggest that oxidative phosphorylation/aerobic respiration genes are modulated by both BAF155 and BAF170, but this modulation does not require Brg1.

2.4 Discussion

The dysregulation of cellular bioenergetics has increasingly been found to be associated with a wide array of human diseases, including cancer, neurological diseases and diabetes [23-26]. Given that oxidative phosphorylation/mitochondrial respiration yields 18 times more ATP per glucose molecule compared to glycolysis, it is conceivable that aerobic respiration is germane to the regulation of cellular bioenergetics in many eukaryotic cells, particularly cells that require a high level of energy supply. One kind of such cells are brain cells. The brain uses

20% of oxygen consumed by the whole human body, even though its mass is only 2% of the whole body. Another kind of such high energy demanding cells are cancer cells. Therefore, understanding the molecular mechanisms governing the regulation of cellular bioenergetics is of fundamental importance to the prevention and treatment of many common diseases.

In this report, we present a new series of experiments to directly characterize the function of Swi3 and its human homologues in the control of aerobic respiration, based on previous indirect experimental and computational evidence suggesting a link between Swi3 and oxygen regulation and aerobic gene expression [27-30]. We found that Swi3 has a novel function in suppressing aerobic respiration genes that are not subjected to glucose repression [3, 6-8]. This Swi3 function is distinct from its function in the SWI/SNF chromatin remodeling complex that enhances transcription of various genes such as the glucose-suppressed *SUC2*, *GAL1* and *GAL10* [18, 31]. First, by analyzing previously identified targets of SWI/SNF proteins, we showed that Swi2 and Swi3 have functions separate from other SWI/SNF components and that Swi3, not Swi2, tends to target genes encoding functions involved in oxidation and reduction or oxidative phosphorylation (Table 2.1).

Table 2.1. The Numbers of Genes Belonging to the Top Five GO Classes of Swi2 and Swi3 Only Targets*

	Total	**response to temperature stimulus**	**vacuolar protein catabolic process**	**response to abiotic stimulus**	**oxidation reduction**	**oxidative phosphorylation**
Swi3	82	11 (0.0003)	8 (0.0006)	13 (0.001)	12 (0.004)	5 (0.006)
	Total	**Ribosome**	**translation**	**rRNA metabolic process**	**maturation of SSU-rRNA**	**RNA binding**
Swi2	63	19 (8.3E-14)	28 (3.6E-10)	11 (0.0004)	7 (0.0004)	16 (0.0007)

*GO analysis was performed using NIH DAVID program. The five GO classes of genes with the lowest p values are shown here. The values in the parentheses are P values.

Next, we showed that Swi3 has a unique function in moderating oxygen consumption in wild type cells, because deletion of the *SWI3* gene, not the *SWI2* gene, caused a substantial increase in oxygen consumption rate (Fig. 2.1). Biochemical analysis showed that the levels of mitochondrial oxidative phosphorylation complexes were substantially increased in *Δswi3* cells, compared to the parent cells (Fig. 2.2). We also measured the promoter activities of two representative genes (*CYC1* and *CYC7*) encoding functions involved in mitochondrial respiration in heme-sufficient and heme-deficient cells. Our results showed that Swi3 strongly impacts heme/oxygen-dependent activation of *CYC1* and *CYC7* promoters while Swi2 impacts only the basal, heme-independent activities of these promoters (Fig. 2.3). Measurement of oxygen consumption rates and cell growth rates (Figs. 2.4 & 2.5) showed that increased expression of aerobic respiration genes is correlated with increased oxygen consumption rate and growth rate in *Δswi3* cells in air. Evidently, a small fraction of Swi3 may act in the SWI/SNF complex to promote transcription, since *Δswi3* cells do not grow much under hypoxia.

Intriguingly, we found that Swi3 homologues in mammalian cells also play a role in moderating aerobic respiration. We identified the unique targets of the Swi3 homologues BAF155 and BAF170, based on the genome-wide ChIP-Seq data from Euskirchen et al. [15] (Table 2.2). We analyzed these targets and found that BAF155 and BAF170 localize to many oxidative phosphorylation/aerobic respiration genes while the Swi2 homologue Brg1 does not. Furthermore, we confirmed the roles of BAF155 and BAF170 in moderating oxygen consumption by detecting the oxygen consumption rate in HeLa cells with BAF155 or BAF170 knocked down (Fig. 2.6).

Table 2.2. Pathway Analysis of Genes Bound by BAF155, BAF170 and/or Brg1*

	[155+170]-Brg1	[155+170+Brg1]	155 only	170 only	Brg1 only
Total	13198	10005	8381	1590	281
Gene Promoters	4891	3737	1540	321	40
KEGG (Genes, p value)	OxP (47, 4.7E-5) CML (32, 2.8E-5) Ins (48, 6.3E-5)	Cancer (86, 6.5E-6) CML (25, 7.2E-4) p53 (24, 3.8E-4)	Ad junc (15, 4.0E-4) HCM (13, 9.0E-3) ARVC (13, 1.0E-2)	Melano (6, 3.0E-2) Endo (8, 4.3E-2) Hedge (4, 7.6E-2)	None

*Shown here are the total number (Total) of genomic regions associated with BAF155 and BAF170, but not Brg1 ([155+170]-Brg1); with BAF155, BAF170, and Brg1 ([155+170+Brg1]); with only BAF155 (155 only); with only BAF170 (170 only); and with only Brg1 (Brg1 only). These regions that are within promoters of genes were identified, and the number of genes (gene promoters) are shown. KEGG pathway analysis was then performed with these groups of genes by using the NIH DAVID program. Three most significant pathways for each group of genes are shown here. The numbers in parentheses indicate the number of genes in the pathway and the corresponding p value. OxP: Oxidative phosphorylation; CML: Chronic myeloid leukemia; Ins: Insulin signaling pathway; Cancer: Pathways in cancer; p53: p53 signaling pathway; Ad junc: Adherens junction; HCM: Hypertrophic cardiomyopathy; ARVC: Arrhythmogenic right ventricular cardiomyopathy; Melano: Melanogenesis; Endo: Endocytosis; Hedge: Hedgehog signaling pathway. No known genes were found to be in regions that are bound by only Brg1.

Together, these experimental and computational data demonstrated that Swi3 and its mammalian homologues is a master regulator in moderating oxygen consumption and oxidative phosphorylation.

Our results provide a novel mechanism for the action of Swi3 human homologues, and perhaps homologues of other SWI/SNF proteins in the pathogenesis of cancer and neurological diseases. The mutations of Swi3 and other SWI/SNF proteins have been shown to be associated with a range of cancers [23, 24]. Aerobic respiration has been increasingly shown to provide critical cellular energy supply in many type of cancer cells [32-36]. Swi3 moderates oxygen consumption and aerobic respiration, thereby limiting cellular energy generation and promoting controlled cellular function. Loss of function of Swi3 would lead to intensified oxygen consumption and cellular energy production, thereby promoting tumorigenic transformation of cellular functions.

Swi3 homologues and other SWI/SNF proteins have been suggested to play important roles in the nervous systems [25, 37]. Neuronal cells have a high number of mitochondria and

continuously require a high level of cellular energy from aerobic respiration. Indeed, several lines of experimental evidence have shown that the Swi3 mammalian homologues BAF155 and BAF170 are critical for brain development and brain function. For example, Kim and colleagues showed that BAF155 is essential for early embryogenesis and plays an important role in brain development in mice [38]. Recently, Tuoc and colleagues showed that BAF170 is an intrinsic factor that controls cortical size and thickness in mice [39, 40]. It has been presumed that this function of BAF155 and BAF170 is attributable to their involvement in the mSWI/SNF complexes, because this has been the well-known biochemical property of these proteins. However, our new finding demonstrating a role of Swi3, BAF155 and BAF170 in oxidative phosphorylation provides a new, likely mechanism for these proteins to impact neuronal functions and brain development. If BAF155 and BAF170 indeed modulate cellular bioenergetics in a variety of eukaryotic cells, their dysfunction may underlie the pathogenesis of a wide range of common diseases, including cancer, neurological diseases and diabetes.

References

1. Haldi, M. and L. Guarente, *N-terminal deletions of a mitochondrial signal sequence in yeast. Targeting information of delta-aminolevulinate synthase is encoded in non-overlapping regions.* J Biol Chem, 1989. **264**: p. 17107-12.

2. Guarente, L., et al., *Distinctly regulated tandem upstream activation sites mediate catabolite repression of the CYC1 gene of S. cerevisiae.* Cell, 1984. **36**(2): p. 503-11.

3. Prezant, T., K. Pfeifer, and L. Guarente, *Organization of the regulatory region of the yeast CYC7 gene: multiple factors are involved in regulation.* Mol Cell Biol, 1987. **7**(9): p. 3252-9.

4. Hon, T., et al., *A Mechanism of Oxygen Sensing in Yeast: Multiple Oxygen-Responsive Steps in the Heme Biosynthetic Pathway Affect Hap1 activity.* J. Biol. Chem., 2003. **278**: p. 50771-80.

5. Kundaje, A., et al., *A predictive model of the oxygen and heme regulatory network in yeast.* PLoS Comput Biol, 2008. **4**(11): p. e1000224.

6. Zhang, L. and L. Guarente, *HAP1 is nuclear but is bound to a cellular factor in the absence of heme.* J Biol Chem, 1994. **269**(20): p. 14643-7.

7. Zhang, L., A. Hach, and C. Wang, *Molecular Mechanism Governing Heme Signaling in Yeast: a Higher-Order Complex Mediates Heme Regulation of the Transcriptional Activator HAP1.* Mol Cell Biol, 1998. **18**(7): p. 3819-28.

8. Guarente, L. and T. Mason, *Heme regulates transcription of the CYC1 gene of S. cerevisiae via an upstream activation site.* Cell, 1983. **32**(4): p. 1279-86.

9. Hon, T., et al., *The heme activator protein Hap1 represses transcription by a heme-independent mechanism in Saccharomyces cerevisiae.* Genetics, 2005. **169**(3): p. 1343-52.

10. Papandreou, I., et al., *HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption.* Cell Metab, 2006. **3**(3): p. 187-97.

11. Gregg, C., P. Kyryakov, and V.I. Titorenko, *Purification of mitochondria from yeast cells.* J Vis Exp, 2009(30).

12. Wittig, I., H.P. Braun, and H. Schagger, *Blue native PAGE.* Nat Protoc, 2006. **1**(1): p. 418-28.

13. Schamel, W.W.A., *Two-dimensional blue native polyacrylamide gel electrophoresis*, in *Current protocols in cell biology*, J.S. Bonifacino, et al., Editors. 2008, John Wiley & Sons, Inc.

14. Hu, Z., P.J. Killion, and V.R. Iyer, *Genetic reconstruction of a functional transcriptional regulatory network.* Nat Genet, 2007. **39**(5): p. 683-7.

15. Euskirchen, G.M., et al., *Diverse roles and interactions of the SWI/SNF chromatin remodeling complex revealed using global approaches.* PLoS Genet, 2011. **7**(3): p. e1002008.

16. Goldberg, A.A., et al., *Effect of calorie restriction on the metabolic history of chronologically aging yeast.* Exp Gerontol, 2009. **44**(9): p. 555-71.

17. Zitomer, R.S., et al., *Elements involved in oxygen regulation of the Saccharomyces cerevisiae CYC7 gene.* Mol Cell Biol, 1987. **7**(6): p. 2212-20.

18. Peterson, C.L. and I. Herskowitz, *Characterization of the yeast SWI1, SWI2, and SWI3 genes, which encode a global activator of transcription.* Cell, 1992. **68**(3): p. 573-583.

19. Da, G., et al., *Structure and function of the SWIRM domain, a conserved protein module found in chromatin regulatory complexes.* Proc Natl Acad Sci U S A, 2006. **103**(7): p. 2057-62.

20. Boyer, L.A., et al., *Essential role for the SANT domain in the functioning of multiple chromatin remodeling enzymes.* Mol Cell, 2002. **10**(4): p. 935-42.

21. Ho, L., et al., *An embryonic stem cell chromatin remodeling complex, esBAF, is essential for embryonic stem cell self-renewal and pluripotency.* Proc Natl Acad Sci U S A, 2009. **106**(13): p. 5181-6.

22. De, S., et al., *Dynamic BRG1 recruitment during T helper differentiation and activation reveals distal regulatory elements.* Mol Cell Biol, 2011. **31**(7): p. 1512-27.

23. DelBove, J., et al., *Identification of a core member of the SWI/SNF complex, BAF155/SMARCC1, as a human tumor suppressor gene.* Epigenetics, 2011. **6**(12): p. 1444-53.

24. Shain, A.H. and J.R. Pollack, *The spectrum of SWI/SNF mutations, ubiquitous in human cancers.* PLoS One, 2013. **8**(1): p. e55119.

25. Ronan, J.L., W. Wu, and G.R. Crabtree, *From neural development to cognition: unexpected roles for chromatin.* Nat Rev Genet, 2013. **14**(5): p. 347-59.

26. Tseng, Y.H., A.M. Cypess, and C.R. Kahn, *Cellular bioenergetics as a target for obesity therapy.* Nat Rev Drug Discov, 2010. **9**(6): p. 465-82.

27. Shah, A.N., et al., *Deletion of a subgroup of ribosome-related genes minimizes hypoxia-induced changes and confers hypoxia tolerance.* Physiol Genomics, 2011. **43**(14): p. 855-72.

28. Henke, R.M., et al., *Hypoxia elicits broad and systematic changes in protein subcellular localization.* Am J Physiol Cell Physiol, 2011. **301**(4): p. C913-C928.

29. Ghosh Dastidar, R., et al., *The nuclear localization of SWI/SNF proteins is subjected to oxygen regulation.* Cell Biosci, 2012. **2**(1): p. 30.

30. Gat-Viks, I., et al., *Understanding gene sequence variation in the context of transcription regulation in yeast.* PLoS Genet, 2010. **6**(1): p. e1000800.

31. Peterson, C.L., A. Dingwall, and M.P. Scott, *Five SWI/SNF gene products are components of a large multisubunit complex required for transcriptional enhancement.* Proc Natl Acad Sci U S A, 1994. **91**(8): p. 2905-8.

32. Whitaker-Menezes, D., et al., *Hyperactivation of oxidative mitochondrial metabolism in epithelial cancer cells in situ: visualizing the therapeutic effects of metformin in tumor tissue.* Cell Cycle, 2011. **10**(23): p. 4047-64.

33. Kaambre, T., et al., *Metabolic control analysis of cellular respiration in situ in intraoperational samples of human breast cancer.* J Bioenerg Biomembr, 2012.

34. Frezza, C., et al., *Metabolic profiling of hypoxic cells revealed a catabolic signature required for cell survival.* PLoS One, 2011. **6**(9): p. e24411.

35. Hooda, J., et al., *Enhanced heme function and mitochondrial respiration promote the progression of lung cancer cells.* PLoS One, 2013. **8**(5): p. e63402.

36. Hooda, J., A. Shah, and L. Zhang, *Heme, an essential nutrient from dietary proteins, critically impacts diverse physiological and pathological processes.* Nutrients, 2014. **6**(3): p. 1080-102.

37. Santen, G.W., M. Kriek, and H. van Attikum, *SWI/SNF complex in disorder: SWItching from malignancies to intellectual disability.* Epigenetics, 2012. **7**(11): p. 1219-24.

38. Kim, J.K., et al., *Srg3, a mouse homolog of yeast SWI3, is essential for early embryogenesis and involved in brain development.* Mol Cell Biol, 2001. **21**(22): p. 7787-95.

39. Tuoc, T.C., et al., *Chromatin regulation by BAF170 controls cerebral cortical size and thickness.* Dev Cell, 2013. **25**(3): p. 256-69.

40. Tuoc, T.C., R. Narayanan, and A. Stoykova, *BAF chromatin remodeling complex: cortical size regulation and beyond.* Cell Cycle, 2013. **12**(18): p. 2953-9.

CHAPTER 3

HEME PROMOTES TRANSCRIPTIONAL ACTIVITY OF GIS1, A MEMBER OF THE HISTONE DEMETHYLASE JMJD2/KDM4 FAMILY

Authors - Sneha Lal and Li Zhang

Department of Biological Sciences

Center for Systems Biology

The University of Texas at Dallas

800 West Campbell Road

Richardson, Texas 75080-3021

Reprint from Nucleic Acids Research, 2017 (Open Access)

- https://doi.org/10.1093/nar/gkx1051

ABSTRACT

The yeast Gis1 protein is a transcriptional regulator belonging to the JMJD2/KDM4 subfamily of demethylases that contain a JmjC domain, which are highly conserved from yeast to humans. They have important functions in histone methylation, cellular signaling, and tumorigenesis. Besides serving as a cofactor in many proteins, heme is known to directly regulate the activities of proteins ranging from transcriptional regulators to potassium channels. Here, we report a novel mechanism of heme regulation of Gis1 transcriptional activity. *In vivo* functional analysis showed that the ZnF, not the JmjN+JmjC domain, promotes heme activation of transcriptional activity. The JmjN+JmjC domain can confer heme activation of transcriptional activity in an unrelated protein. These results demonstrate that Gis1 represents a novel class of heme sensing and signaling proteins, and that heme binding to ZnF stimulates Gis1 transcriptional activity.

3.1 Introduction

Many JmjC domain-containing proteins possess demethylase activity and can remove specific methyl groups on histones or other proteins. They are dioxygenases that use α-ketoglutarate and Fe^{2+} to oxidize various substrates [1-3]. In humans, 32 JmjC domain-containing proteins have been identified [4]. These proteins have fundamental biological functions, and their dysfunctions are implicated in many pathological processes, including developmental deficiency, cancer, and cardiovascular diseases [4-6]. Likewise, heme, iron protoporphyrin IX, plays key physiological and pathological roles in virtually all living organisms [7]. As an essential prosthetic group and cofactor in many proteins and enzymes, heme is required for the proper functioning of the mitochondrial respiratory chain complexes; the synthesis and sensing of CO and NO; and the activity of many enzymes involved in the transport, storage, and utilization of oxygen, such as cytochrome c and P450s [8-10]. Further, heme serves as an important signaling molecule that directly regulates diverse molecular and cellular processes ranging from gene transcription and translation to microRNA processing and potassium channel activation [11-16]. Recent epidemiological and experimental studies have implicated altered heme availability in the development and progression of an array of common human diseases, including cancer, diabetes, and cardiovascular diseases [17, 18].

Previously, the yeast protein Gis1 was shown to be a transcriptional regulator also belonging to the JMJD2/KDM4 subfamily of demethylases [19-21]. Gis1 is highly homologous to the mammalian JMJD2/KDM4 proteins in the JmjN+JmjC domain possessing demethylase activity [19]. These proteins play important roles in histone methylation, oxygen regulation, and hormonal signaling [3, 5, 6, 22]. Gis1 binds to the PDS (post-diauxic shift) element [23, 24] and

modulates the transcription of hundreds of genes involved in nutrient signaling, oxidative stress signaling, and aging [25-30]. Gis1 can both activate and repress transcription [24, 31]. Additionally, several studies have shown that Gis1 possesses weak to moderate demethylase activity towards histone H3 [20, 21, 32]. Gis1 contains multiple domains: a JmjN region, a JmjC region, a coiled-coil domain, two C2H2 type zinc fingers (ZnFs), and two transcription activation domains (TADs, Fig. 3.1A) [28, 33, 34]. JmjN and JmjC interact physically to form a structural unit or a domain [34]. The JmjN+JmjC domain presumably confers histone demethylase activity, but is dispensable for transcriptional activation by Gis1 [33]. Intriguingly, a survey of the protein sequence identified a CP motif, also known as heme regulatory motif (HRM), in the JmjC domain and another one in the C2H2 ZnF (Fig. 3.1A). While a wide array of peptides containing HRM or CP motifs bind to heme reversibly in the micromolar range [35, 36], the existence of HRMs in proteins per se does not necessarily indicate heme binding by the proteins or heme responsiveness in protein activity. For example, only one or some of the CP motifs in transcriptional regulators Hap1 and Bach1 are essential for heme regulation [7, 35, 37-39]. Structural environment dictates whether CP motifs play a role in heme regulation [40]. Direct biochemical and functional studies are necessary to determine heme binding and heme regulation of interested proteins. Notably, Gis1 is oxygen sensitive, and oxygen signaling can be mediated by heme [41-43]. We therefore explored the possibility that Gis1 activity is regulated by heme.

Indeed, we found that Gis1 transcriptional activity is regulated by heme, although heme is not required for Gis1 nuclear localization. The JmjN+JmjC domain is not required for heme activation of Gis1 activity, but confers heme activation via an unrelated protein. Additionally, we showed that heme does not affect Gis1 DNA binding *in vitro*. We found that the Gis1 regions

mediating heme binding and regulation can confer heme regulation via an unrelated DNA-binding domain. These results demonstrated that Gis1 is a novel, multi-functional signaling protein, which senses intracellular heme levels and modulates its transcriptional and demethylase activities.

3.2 Materials and Methods

3.2.1 Yeast strains and plasmids

The yeast strains used were BY4741Gis1-GFPΔhem1 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hem1-Δ100 GIS1-GFP), MHY101 (MATa ura3-52 leu2-3,112 his4-519 ade1-100 hem1-Δ100 URA3::PDS-lacZ), and MHY101Δgis1 (MATa ura3-52 leu2-3,112 his4-519 ade1-100 hem1-Δ100 URA3::PDS-lacZ gis1::LEU2 ura3::Kan^r). To delete the GIS1 gene, MHY101 cells were transformed with a PCR product containing LEU2 gene in the middle and 44 bps sequences flanking the open reading frame sequence of GIS1 on both sides. Knockout strains were confirmed by PCR and β-galactosidase assay. The PDS element-driven LEU2-lacZ reporter was introduced by transforming yeast strains with the with linearized, NcoI-cut pLS9-PDS plasmid, as described previously [24]. In the MHY101Δgis1 strain, the URA3 gene was deleted by transformation with a PCR product containing Kan^r sequence in the middle and 44 bps of sequence flanking the open reading frame sequence of URA3 on both sides. The HEM1 gene in the BY4741Gis1-GFP strain was deleted as described previously [44].

The PDS element-driven LEU2-lacZ reporter plasmid pLS9-PDS is as described [24], and was provided by Dr. Claudio de Virgilio's lab (University of Fribourg, Switzerland). The yeast expression vectors for full-length Gis1 (pYY53), Gis1ΔJmjC (pYY54), and Gis1ΔZnF (pYY55) are as described [33], and were provided by Dr. Rolf Sternglanz's lab (Stony Brook

University, New York). The expression vectors for Gis1ΔJmjN, Gis1ΔJmjN/C, and Gis1ΔJmjN/C/CC are as described [34, 45], and were provided by Dr. Nianshu Zhang's lab (University of Cambridge, UK). The expression vectors for fusion proteins HG1, HG2, $HG2_{mut}$, HG3, were constructed by replacing the Hap1-coding sequences in the yeast expression vector SD5-HAP1 [46] with corresponding Gis1-coding sequences. The DNA containing the coding sequences for fusion proteins was generated by overlapping PCR, as described previously [47]. Other inactive fusion proteins containing the Hap1 DNA-binding domain and various Gis1 regions were constructed in the same way. Gis1 mutants with point mutations were generated by using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technology). The sequences of fusion proteins were confirmed by DNA sequencing by Eurofins MWG operon USA (Louisville, KY).

3.2.2 Cell growth, β-galactosidase assays, and immunofluorescence staining

Yeast cells were grown in rich YPD or synthetic complete media, as described previously [48, 49]. Cell density was determined by measuring optical density at 600 nm. To determine β-galactosidase levels from reporter genes in *Δhem1* cells bearing the PDS element-driven *LEU2-lacZ* reporter or the Hap1-driven UAS1-*CYC1-lacZ* reporter, cells were grown in synthetic complete medium containing a limiting amount of the heme precursor 5-aminolevulinate (2.5 μg/ml) or a high amount of 5-aminolevulinate (250 μg/ml). Cells were collected after diauxic shift for measuring Gis1 activity or after they reached $A_{1.0\text{-}1.5}$ for measuring Hap1 activity. Collected cells were then subjected to chloroform permeabilization and β-galactosidase assays. The activities were measured and calculated in Miller units, as described previously [50, 51]. For immunofluorescence staining, cells bearing the expression vector for FLAG-tagged Gis1 or

deletion proteins were grown in synthetic complete media to $A_{0.8\text{-}1.0}$. Subsequent fixing and staining of cells were carried out as described [52, 53]. Briefly, cells were fixed with 5% formaldehyde for 90 min and permeabilized with Zymolyase. Cells were incubated with an anti-FLAG antibody overnight at room temperature, and then incubated with an affinity-purified, fluorescein-conjugated anti-rabbit IgG goat antibody for 2 h. Finally, cells were stained with DAPI for 10 min before viewing and imaging.

3.2.3 Preparation of yeast extracts, Western blotting, electrophoretic mobility shift assays, and ChIP-qPCR

For preparation of yeast cell extracts, *Δgis1* cells bearing the expression vector for full-length Gis1 or Gis1 deletion proteins were grown in synthetic complete media to $A_{1.0\text{-}1.5}$. Cells were harvested and extracts were prepared as described previously [54]. Protein concentrations were determined with the BCA assay kit (Thermo Scientific). For Western blotting, 75 μg of proteins from each treatment condition were electrophoresed on 10% SDS–Polyacrylamide gels, and then transferred onto the Immuno-Blot PVDF Membrane (Bio-Rad). The membranes were probed with antibodies, followed by detection with a chemiluminescent Western blotting kit (Roche Diagnostics). The signals were detected with a Carestream image station 4000MM Pro, and quantitation was performed with the Carestream molecular imaging software version 5.0.5.30 (Carestream Health, Inc.).

For EMSAs, DNA-binding reactions were carried out in a 20 μl volume with 5% glycerol, 4 mM Tris (pH 8), 40 mM NaCl, 4 mM $MgCl_2$, 10 mM DTT, 3 μg of salmon sperm DNA, and 300 μg of bovine serum albumin per ml in the presence or absence of 2μM heme.

Approximately 0.01 pmol of labeled UAS1/*CYC1* or PDS and 20 μg of protein extracts were used in each reaction. The reaction mixtures were incubated at room temperature for 1 h and then loaded onto 4% polyacrylamide gels in 1/3 X Tris-borate-EDTA for polyacrylamide gel electrophoresis (PAGE) at 4°C. For antibody super shifts of the DNA-protein complexes, anti-Hap1 or anti-FLAG antibodies or pre-immune serum was added to the reaction and were incubated for 40 min, followed by electrophoresis on 4% nondenaturing polyacrylamide gels.

Chromatin immunoprecipitation (ChIP) was carried out as described previously [51]. Briefly, yeast MHY101*Δgis1* cells bearing the expression vector for full-length Gis1-FLAG or an empty vector were grown to $A_{1.2}$ under heme-deficient or heme-sufficient conditions. Cells were collected and fixed with formaldehyde for 30 min at room temperature and then resuspended in lysis buffer. Cells were permeabilized by agitation with equal volumes of glass beads. Permeabilized cells were sheared using a sonicator. Cell extracts were collected and incubated with magnetic beads (Dynabeads Protein G, Novex Thermo Fisher) bound with or without anti-FLAG antibodies (Sigma). Bound complexes were eluted using elution buffer. Subsequently, crosslinking was reversed by incubating with 1% SDS at 65 ℃ overnight. DNA was collected and subjected to qPCR analysis (LightCycler FastStart DNA MasterPlus SYBR Green I, Roche) using primers for GRE1 promoter (FW 5' GGTTCCAGGTATGGGTTTGA, RV 5' ACAACTAAGGCAAAACTGCC). Percentage of input was calculated using the ΔCt formula: $(2^{-(\text{Ct IP—Ct Input X DF})})\text{x } 100$.

3.3 Results

3.3.1 Heme is not required for nuclear localization of Gis1

To determine if heme is required for the nuclear localization of Gis1, we imaged live cells expressing Gis1-GFP from the chromosomal location of the *GIS1* gene [56], we found that (Fig. 3.1B) Gis1 localizes entirely to the nucleus even in heme-deficient cells.

Transcriptional activators are modular proteins with at least two separate domains, a DNA-binding domain and a transcription activation domain (TAD, e.g., see Fig 3.1A), which mediate DNA binding and interaction with transcriptional machinery necessary for transcription to be activated, respectively and often independently [57].

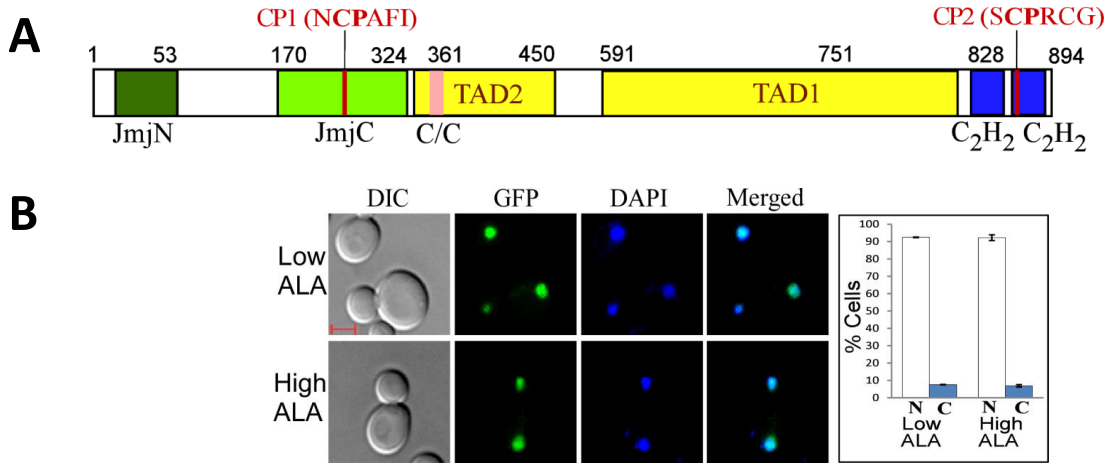


Figure 3.1. (A) The domain structure of Gis1 protein. Shown here are the previously identified JmjN+JmjC domain, coiled-coil domain (C/C), two C2H2 type zinc fingers, and two transcription activation domains (TAD1 and TAD2). Also shown are two CP motifs. (B) Images showing the effect of heme on subcellular localization of Gis1. DIC, GFP, DAPI and merged fluorescent images of cells expressing Gis1-GFP, grown in the presence of a low (Low) or high (High) level of heme precursor 5-aminolevulinic acid (ALA). The number of cells showing GFP-tagged proteins in the nucleus (N) or cytosol (C) was counted and plotted here. The scale bar represents 1μm.

As such, heme may activate Gis1 by stimulating DNA binding and/or by stimulating TAD activity. To determine whether heme affects DNA binding by Gis1, we performed

electrophoretic mobility shift assays (EMSAs) using purified Gis1 from *E. coli* and Gis1 in yeast extracts prepared from cells expressing Gis1 with a FLAG. Notably, addition of anti-FLAG antibody super-shifted the Gis1-DNA band, showing that the detected band indeed represents Gis1 protein binding. For both purified Gis1 and yeast Gis1, heme did not affect DNA binding (see lanes 1-5, Fig 3.2A and lanes 2-6, Fig. 3.2B).

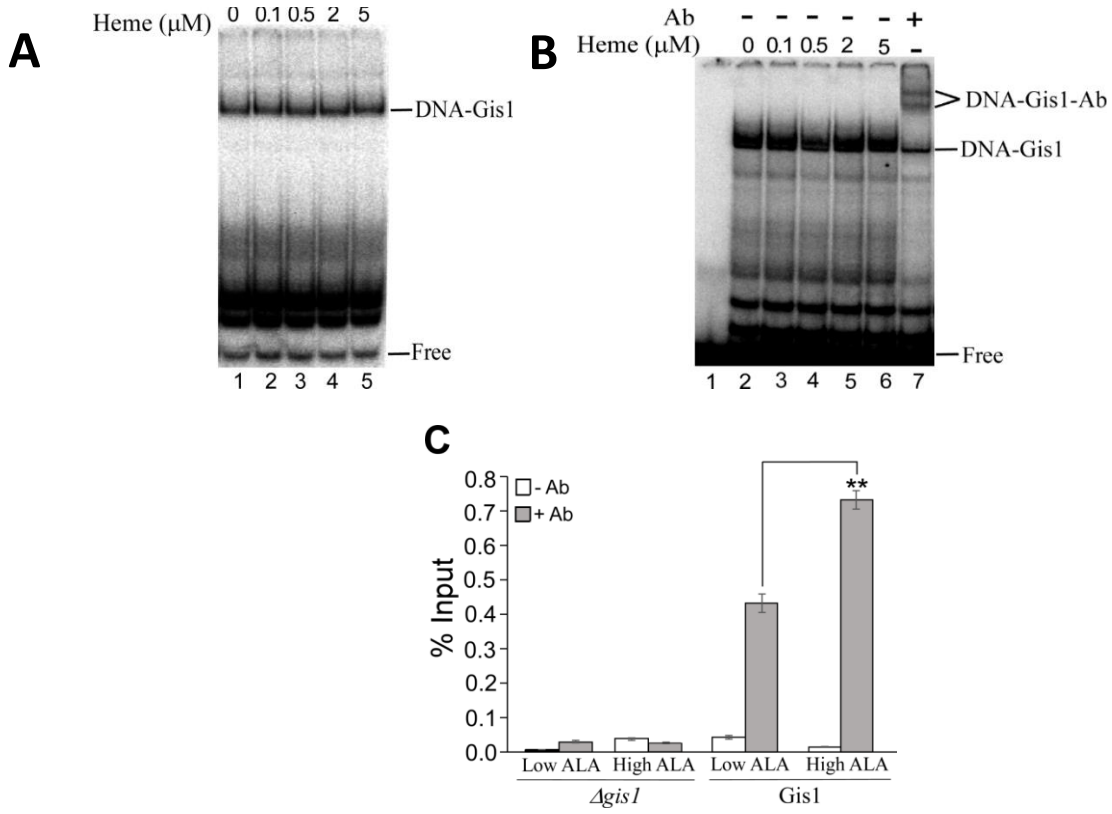


Figure 3.2. Heme does not affect Gis1 DNA binding by purified Gis1 (A) or Gis1 in extracts prepared from yeast cells expressing Gis1 (B). DNA-binding reactions were carried with purified Gis1 or proteins extracted from cells expressing full-length Gis1 with a C-terminal FLAG tag in the presence of increasing concentrations of heme. An anti-FLAG antibody was included in lane 9 for super shift. Lane 1: free probe. (C) Heme does not affect significantly Gis1 binding to the *GRE1* promoter *in vivo*. ChIP-qPCR analysis of Gis1 binding to the GRE1 promoter in heme-deficient (low ALA) and heme-sufficient (high ALA) cells was carried out as described in Materials and Methods. Data from three independent experiments were calculated and plotted here. For statistical analysis, the levels in heme-deficient cells were compared to the levels in heme-sufficient cells, with a Welch 2-sample t-test. **, p value <0.005.

To ascertain the effect of heme on Gis1 DNA binding *in vivo*, we carried out chromatin immunoprecipitation coupled with quantitative PCR (ChIP-qPCR). Data in Fig. 3.2C suggests that heme only slightly stimulated (<2-fold) Gis1 DNA binding to DNA *in vivo*. These results together suggest that enhanced DNA binding does not contribute significantly to heme activation of Gis1 transcriptional activity.

3.3.2 The ZnF, not the JmjN+JmjC domain, is essential for heme activation of Gis1 transcriptional activity

To determine if heme affects Gis1 transcriptional activity, we detected the activities of different Gis1 deletion mutants and full-length Gis1 (for control and comparison) in heme-deficient and heme-sufficient cells. We examined whether heme modulates Gis1 transcriptional activity using a PDS (post-diauxic shift) element-driven *lacZ* reporter [24]. Gis1 binds to PDS and activates genes induced by nutrient limitation following diauxic shift, such as *GRE1* [24, 45, 55]. We measured the reporter activity in heme-deficient and heme-sufficient cells following diauxic shift. We found that a high level of intracellular heme was required for the activation of Gis1 transcriptional activity (Fig. 3.3A). ΔN/C (with the JmjN+JmjC domain deleted) exhibited somewhat enhanced stimulation by heme when compared to full-length Gis1 (see Fig. 3.3A). A previous study showed that Gis1 is negatively regulated by proteolysis [45]. This is consistent with our data shown in Fig. 3.3: the deletion protein with lower protein level (Fig. 3.3C: ΔN/C/CC) exhibited lower transcriptional activity in heme-sufficient cells (Fig. 3.3A). However, the slight differences in the protein levels of full length Gis1 and ΔN/C between heme-sufficient and heme-deficient conditions cannot account for much greater differences in transcriptional

activities (compare Fig. 3.3A with 3.3C). Also, immunofluorescence staining showed that full-length Gis1 and ΔN/C and were nuclear (Fig. 3.4). These results show that heme activation of Gis1 transcriptional activity is not fully attributable to increased Gis1 protein levels and that the JmjN+JmjC domain is dispensable for heme activation.

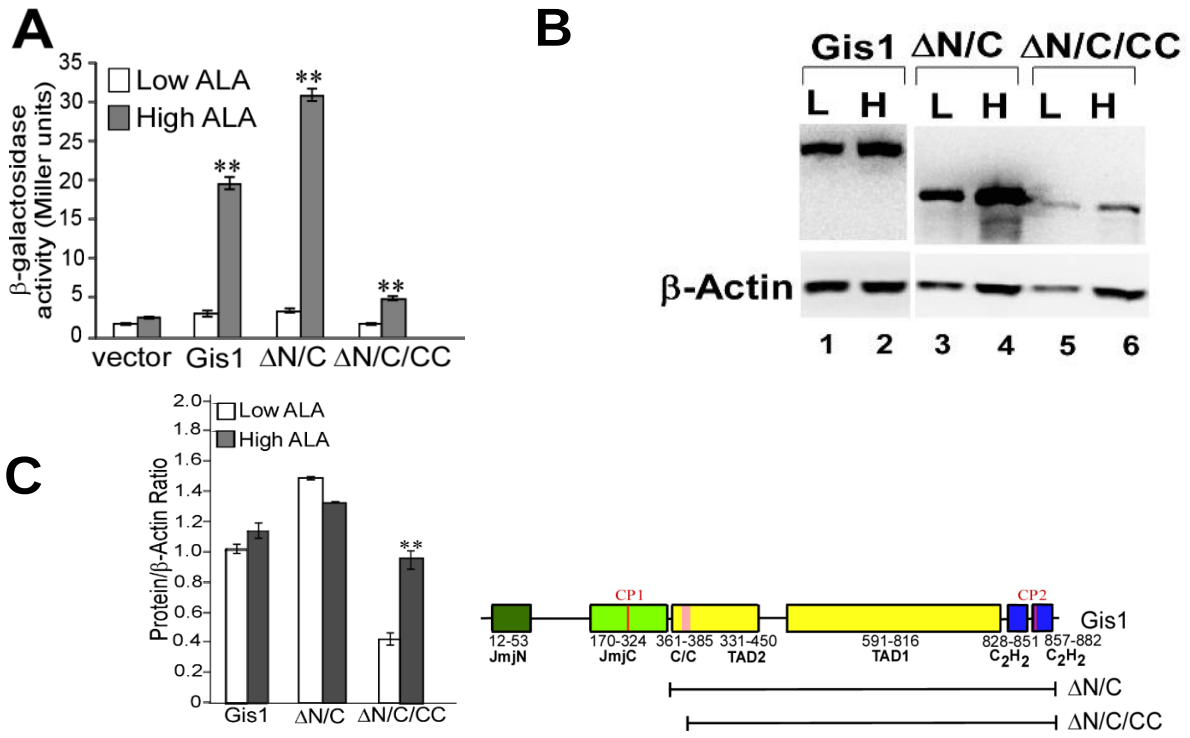


Figure 3.3. The transcriptional activities and protein levels of various Gis1 deletion proteins under heme-deficient and heme-sufficient conditions. (A) The Gis1 JmjN+JmjC domain is dispensable for heme activation of Gis1 transcriptional activity. Yeast *Δgis1Δhem1* cells bearing the empty vector or the expression vector for full-length Gis1, deletion mutant ΔN/C (JmjN+JmjC deleted, see Fig. 3.1A for Gis1 domains) or ΔN/C/CC (JmjN+JmjC & C/C deleted) under the control of its native promoter, and the PDS-*lacZ* reporter were grown in the presence of a low (2.5 μg/ml) or high (250 μg/ml) level of 5-aminolevulinc acid (ALA) until post diauxic shift for Gis1 to be activated. Then, cells were collected and β-galactosidase activities were measured. The plotted values are averages from at least three independent cultures. (B & C) Gis1 protein levels in heme-deficient and heme-sufficient cells. Yeast cells were grown as described in (A), and cell extracts were prepared and subjected to Western blotting (B). The protein levels were quantified and plotted in (C). For statistical analysis, the levels in heme-deficient cells were compared to the levels in heme-sufficient cells, with a Welch 2-sample t-test. **, p value <0.005.

To define Gis1 residues mediating heme activation of Gis1 transcriptional activity, we engineered two fusion proteins: HG1, containing Gis1 residues 386-828 (lacking ZnF; see Fig. 3.1A for Gis1 domains) and an unrelated DNA-binding domain (Hap1 residues 1-225, no CP motif present); and HG2, containing Gis1 residues 386-894 (with the ZnF) and the Hap1 DNA-binding domain. Fig. 3.5A shows that both fusion proteins exhibited high transcriptional activity at the Hap1-driven UAS1-*CYC1-lacZ* reporter in heme-deficient cells.

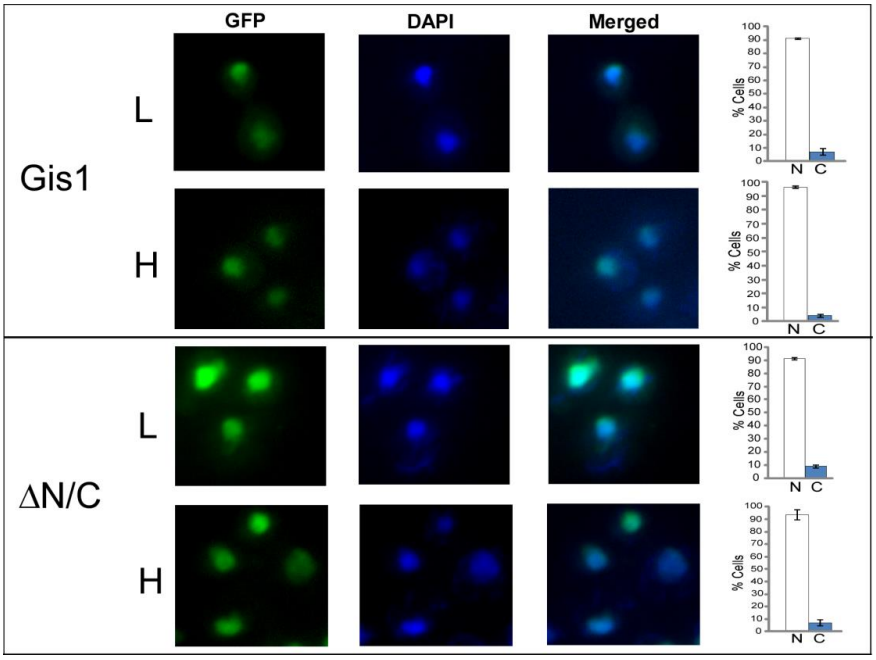


Figure 3.4. The nuclear localization of Gis1 and deletion proteins is not heme dependent. Yeast cells expressing FLAG-tagged full-length Gis1 or ΔN/C proteins were grown under heme-deficient (L) or heme-sufficient (H) conditions. Cells were subjected to indirect immunofluorescence staining. The number of cells showing FLAG-tagged proteins in the nucleus (N) or cytosol (C) was counted and plotted here. The scale bar represents 1μm.

Interestingly, heme further activated the activity of HG2, but not HG1. Note that other fusion proteins, including those containing full-length Gis1 or Gis1 lacking ZnF, were labile and inactive *in vivo*, likely because altered domain architecture disrupts protein function. Further,

when CP2 was mutated (Cys to Ala, HG2C859A, Fig. 3.5A), the degree of heme activation was reduced. The data clearly show that ZnF is essential for heme activation of Gis1 transcriptional activity. Additionally, by performing EMSAs with yeast extracts prepared from cells expressing fusion proteins, we found that HG1, HG2, and HG2C859A all bind to the Hap1-binding site strongly, independently of heme (Fig. 3.5B), as expected.

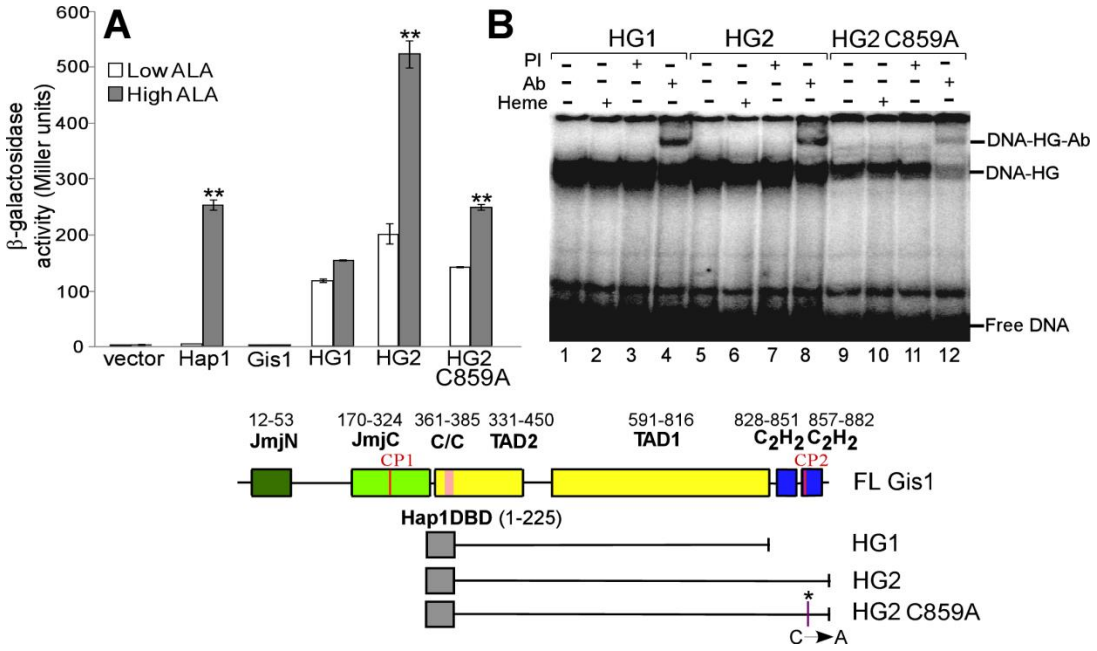


Figure 3.5. (A) The ZnF is essential for heme activation of Gis1 transcription-activating activity. Yeast *Δgis1Δhem1* cells bearing the empty vector or the expression vector for Hap1, Gis1, HG1 (containing Hap1 DNA-binding domain residues 1-225 and Gis1 residues 386-828), HG2 (containing Hap1 residues 1-225 and Gis1 residues 386-894), or HG2C859A (HG2 with the Cys residues in the CP2 motif changed to Ala), and the Hap1-binding UAS1-*CYC1-lacZ* reporter were grown in the presence of a low (2.5 μg/ml) or high (250 μg/ml) level of 5-aminolevulinc acid (ALA). Then, cells were collected and β-galactosidase activities were measured. For statistical analysis, the levels in heme-deficient cells were compared to the levels in heme-sufficient cells, by using Welch 2-sample t-test. **, p value <0.005. (B) Heme does not affect DNA binding by fusion proteins. EMSAs were performed by using extracts prepared from cells expressing HG1 (lanes 1-4), HG2 (lanes 5-8) and HG2C859A (lanes 9-12), respectively, as described in Materials and Methods. Anti-Hap1 antibodies were added in lanes 4 & 8, while pre-immune serum (PI) was included in lanes 3 & 7.

We confirmed that the bands contain the fusion proteins by super shifting with anti-Hap1 antibodies (see lanes 4, 8 & 12, Fig. 3.5B). Together, the results show that the Gis1 ZnF domain promotes heme activation of transcriptional activity, independently of DNA binding.

3.3.3 The Gis1 JmjN+JmjC domain can mediate heme regulation when fused to an unrelated transcriptional activator

To test whether heme binding to the JmjN+JmjC domain can be of functional relevance, we inserted the domain into a minimal transcription activator containing only the Hap1 DNA-binding and activation domains but lacking any heme regulatory regions (HG3, Fig. 3.6A).

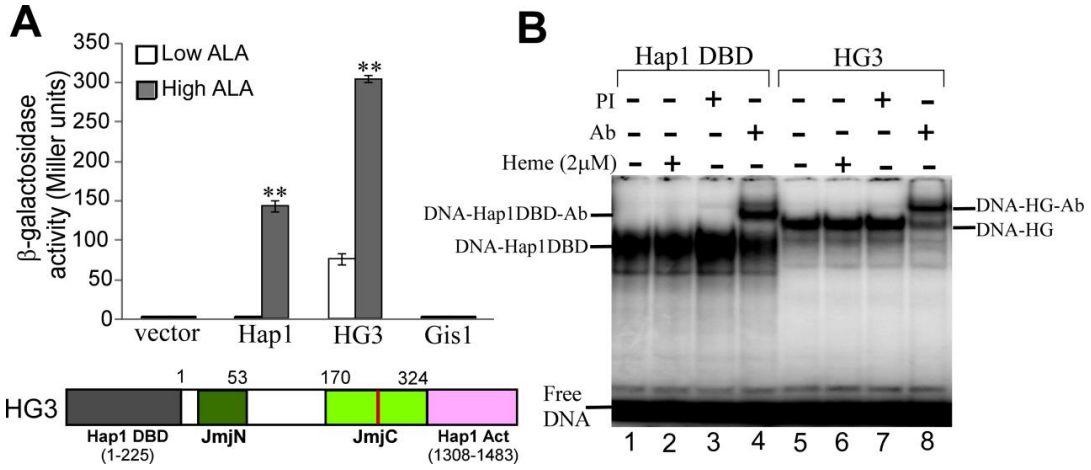


Figure 3.6. The JmjN+JmjC domain can confer heme regulation of transcriptional activity via an unrelated transcription factor. (A) Heme potentiates the transcriptional activity of Hap1-Gis1 fusion containing the Gis1 JmjN+JmjC domain. Yeast *Δgis1Δhem1* cells bearing the empty vector or the expression vector for wild type Hap1 or HG3 (containing Hap1 DNA-binding domain residues 1-225, Gis1 residues 1-324, and Hap1 residues 1308-1483), and the Hap1-binding UAS1-*CYC1-lacZ* reporter were grown in the presence of a low (2.5 μg/ml) or high (250 μg/ml) level of 5-aminolevulinc acid (ALA). Then, cells were collected and β-galactosidase activities were measured. For statistical analysis, the levels in heme-deficient cells were compared to the levels in heme-sufficient cells, by using Welch 2-sample t-test. **, p value <0.005. (B) Heme does not affect DNA binding by the fusion protein. EMSAs were performed by using extracts prepared from cells expressing the Hap1 DNA-binding domain (DBD, lanes 1-4) or the fusion protein HG3 (lanes 5-8), as described in Materials and Methods. Anti-Hap1 antibodies were added in lanes 4 & 8, while pre-immune serum (PI) was included in lanes 3 & 7.

As a control, we showed that the activity of full-length Hap1 is strongly activated by heme, as expected (Fig. 3.6A) [58]. Strikingly, while HG3 exhibited significant transcriptional activity in heme-deficient cells, like HG1 and HG2 (Fig. 3.5A), its activity was further stimulated 4-fold in heme-sufficient cells. This result shows that the JmjN+JmjC domain can confer heme activation via an unrelated protein. Additionally, we showed that HG3 binds to DNA, as expected, and that its DNA-binding activity was not affected by heme (Fig. 3.6B).

3.4 Discussion

3.4.1 Gis1 is a versatile signal transducer

The *GIS1* gene was initially isolated as a multi-copy suppressor of a snf1/mig1/srb8 triple mutant [59]. Gis1 was later found to be a key regulator promoting gene transcription after glucose depletion during and after diauxic shift, when cells shift their metabolism from glucose fermentation to oxidation of ethanol [24]. During diauxic shift, the expression of over 2000 genes is affected [25]. Gis1 has been shown to act both as a transcriptional activator and a repressor [31, 55]. Gis1 acts primarily on genes with PDS elements [30]. These include many genes involved in stress response, phosphate metabolism, sulfur metabolism, amino acid metabolism, ribosome biogenesis, and nutrient signaling [25-30]. Gis1 and Msn2/4 are known to cooperatively mediate the entire Rim15-dependent transcriptional response during the diauxic shift [25]. Through Rim15, Gis1 promotes nutrient signaling via both the TOR and PKA pathways [25, 60]. Additionally, it was suggested that Sch9 can directly act on Gis1 to promote its transcriptional activity [26]. Thus, Gis1 is clearly a key signal transducer mediating signaling by major nutrient signaling pathways. Here, our data show that heme, a key metabolite in oxidative phosphorylation and mitochondrial respiration, directly regulates Gis1 activity. This

function enables Gis1 to coordinate heme signaling with nutrient signaling. The dependence of Gis1 activity on heme fits well with the important function of Gis1 in promoting the transition of cells from glucose fermentation to ethanol oxidation during the diauxic shift.

3.4.2 Structural environment impacts heme binding to Gis1 ZnF and JmjN+JmjC domains and heme regulation

C2H2 zinc finger proteins likely constitute the largest family of regulatory proteins in mammals [61-64]. They can interact with DNA, RNA, and proteins, and modulate an array of biological processes, such as development, differentiation, and tumor suppression [65, 66]. The ZnF enables Gis1 to bind to DNA, and is essential for transcriptional activation by Gis1. Remarkably, the ZnF conferred substantial heme regulation via an unrelated DNA binding domain (Fig. 3.5A). Likewise, the JmjN+JmjC domain conferred substantial heme regulation in an unrelated transcription activator (Fig. 3.6A). Evidently, regardless of the functions of these protein domains in Gis1, their capability to bind heme and alter their conformation enables them to mediate heme regulation when protein architecture is properly constructed, as in the cases of HG2 and HG3 (Figs. 3.5A & 3.6A). In many other cases where protein architecture is not compatible with stable protein domain structure and folding, fusion or mutant proteins are often labile and inactive, as in the cases of fusion protein containing the Hap1 DNA-binding domain and Gis1 with or without ZnF, as well as full-length Gis1 mutants with a mutated Cys residue in the CP motifs (not shown). Previous data have shown that structural environment is crucial for heme binding by CP motifs and dictates whether heme binding causes any changes in protein functions [7, 37-40]. Note that strong heme-binding affinity of protein motifs does not generally correlate with high functional importance in heme signaling and regulation. In the case of Hap1,

six clustered CP motifs with strong heme-binding affinity has no role in heme regulation while one CP motif is essential for heme regulation [35], [39]. Evidently, our data here also suggest that a module with weaker heme-binding affinity is required for heme regulation.

Intriguingly, while the Gis1 JmjN+JmjC domain can promote heme regulation via an unrelated protein (Fig. 3.6), it does not promote heme activation of Gis1 activity (Fig. 3.3A & 3.7). As such, one may ask what function heme binding to the JmjN+JmjC domain may serve. One intriguing possibility is that Gis1 may use the iron ion in heme, not a free iron ion, to catalyze H3K36 demethylation. Further structural studies should uncover the detailed molecular events promoting the multiple fascinating functions of various Gis1 ZnF and JmjN+JmjC domains.

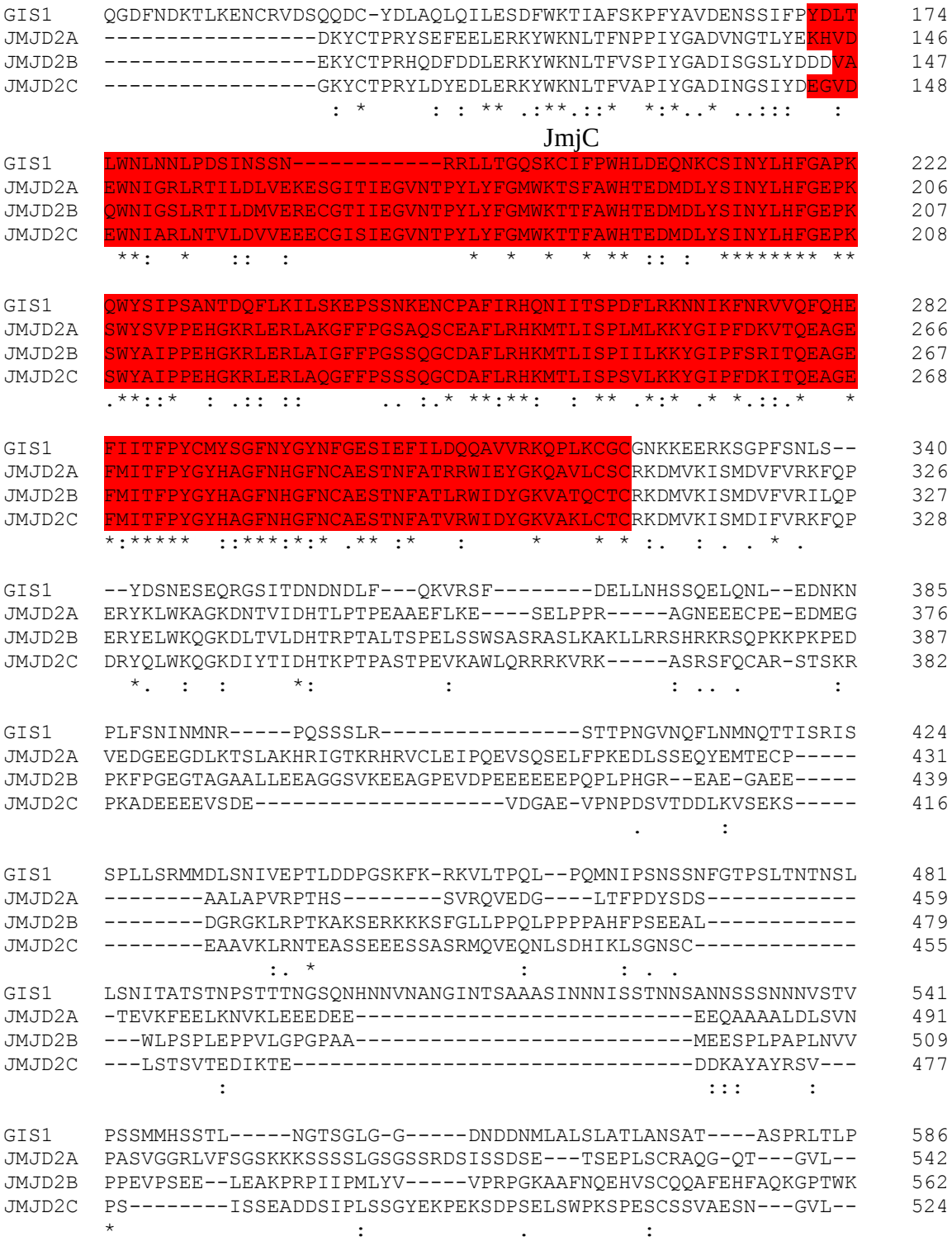
3.4.3 Gis1 represents a novel class of heme sensing and signaling regulator

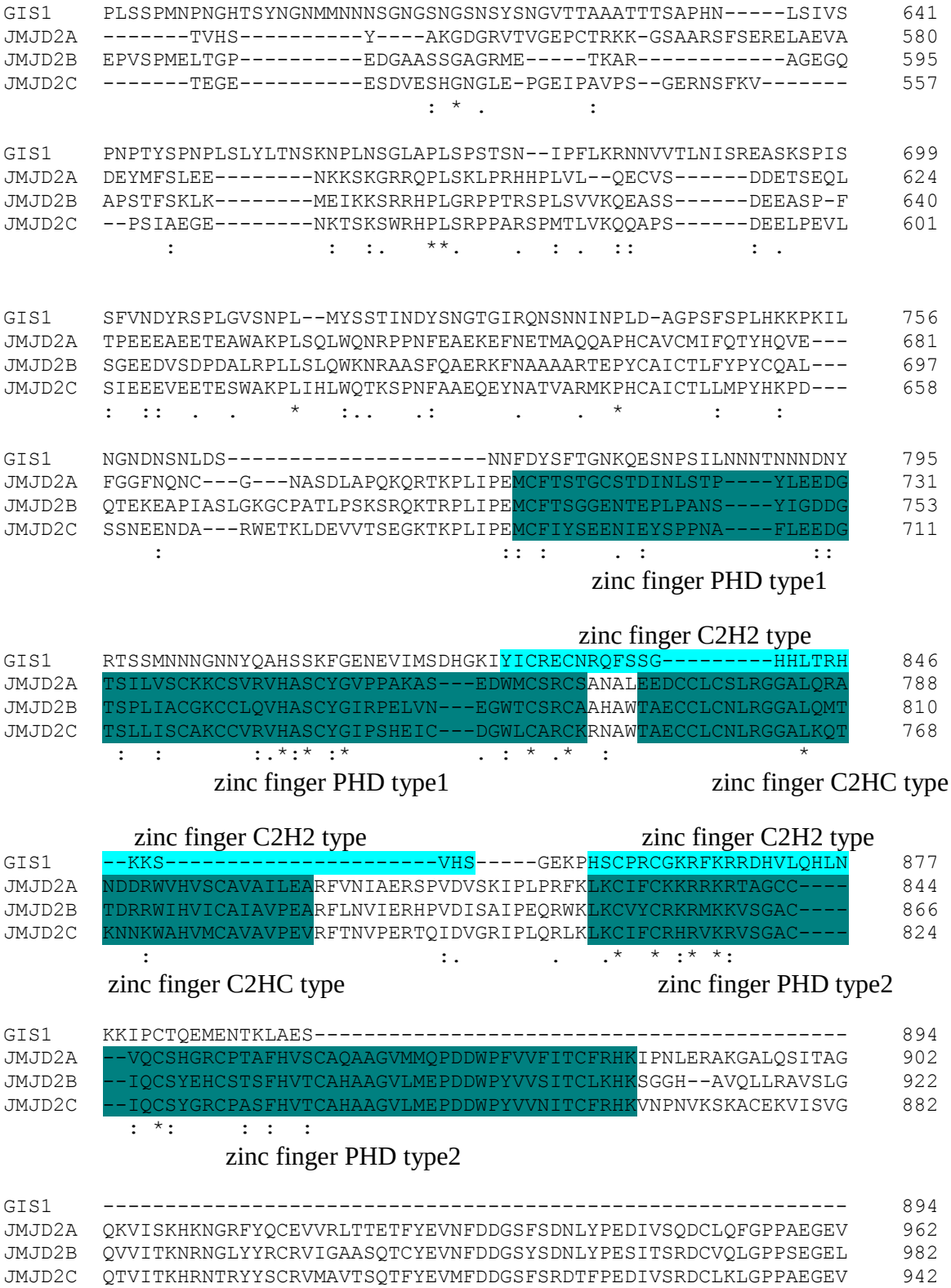
Our biochemical and *in vivo* functional data provide strong experimental evidence showing that heme promotes transcriptional activity of Gis1. Gis1 transcriptional activity is very low in heme-deficient cells (Fig. 3.1B). The mammalian orthologues of Gis1 include JMJD2A (KDM4A), JMJD2B (KDM4B), and JMJD2C (KDM4C) [67]. They possess the JmjN and JmjC domains and PHD type zinc fingers (Fig. 3.7).

JmjN

```
GIS1    ----MEIKPVEVIDGVPVFKPSMMEFANFQYFIDEITKFGI-ENGIVKVIPPKEWLELLE    55
JMJD2A  --MASESETLNPSARIMTFYPTMEEFRNFSRYIAYIESQGAHRAGLAKVVPPKEWKPRAS    58
JMJD2B  -MGSEDHGAQNPSCKIMTFRPTMEEFKDFNKYVAYIESQGAHRAGLAKIIPPKEWKPRQT    59
JMJD2C  MEVAEVESPLNPSCKIMTFRPSMEEFREFNKYLAYMESKGAHRAGLAKVIPPKEWKPRQC    60
                  :    : .* *:* ** :*. ::  : . *  . *:.*::*****

GIS1    GSPPAESLKTIQLDSPIQQQAKRWDKHENGVFSIENEYDNKSYNLTQWKNLAESLDSRIS    115
JMJD2A  ----YDDIDDLVIPAPIQQLVT----GQSGLFTQYN-IQKKAMTVREFRKIANS------    103
JMJD2B  ----YDDIDDVVIPAPIQQVVT----GQSGLFTQYN-IQKKAMTVGEYRRLANS------    104
JMJD2C  ----YDDIDNLLIPAPIQQMVT----GQSGLFTQYN-IQKKAMTVKEFRQLANS------    105
             :.:. : : :**** ..     :.*:*:  *  ::*: .: :::.:*:*
```


JmjC


GIS1 PLSSPMNPNGHTSYNGNMMNNNSGNGSNGSNSYSNGVTTAAATTTSAPHN-----LSIVS 641
JMJD2A -------TVHS----------Y----AKGDGRVTVGEPCTRKK-GSAARSFSERELAEVA 580
JMJD2B EPVSPMELTGP----------EDGAASSGAGRME-----TKAR-----------AGEGQ 595
JMJD2C -------TEGE----------ESDVESHGNGLE-PGEIPAVPS--GERNSFKV------- 557
GIS1 PNPTYSPNPLSLYLTNSKNPLNSGLAPLSPSTSN--IPFLKRNNVVTLNISREASKSPIS 699
JMJD2A DEYMFSLEE--------NKKSKGRRQPLSKLPRHHPLVL--QECVS------DDETSEQL 624
JMJD2B APSTFSKLK--------MEIKKSRRHPLGRPPTRSPLSVVKQEASS------DEEASP-F 640
JMJD2C --PSIAEGE--------NKTSKSWRHPLSRPPARSPMTLVKQQAPS------DEELPEVL 601
GIS1 SFVNDYRSPLGVSNPL--MYSSTINDYSNGTGIRQNSNNINPLD-AGPSFSPLHKKPKIL 756
JMJD2A TPEEEAEETEAWAKPLSQLWQNRPPNFEAEKEFNETMAQQAPHCAVCMIFQTYHQVE--- 681
JMJD2B SGEEDVSDPDALRPLLSLQWKNRAASFQAERKFNAAAARTEPYCAICTLFYPYCQAL--- 697
JMJD2C SIEEEVEETESWAKPLIHLWQTKSPNFAAEQEYNATVARMKPHCAICTLLMPYHKPD--- 658
GIS1 NGNDNSNLDS--------------------NNFDYSFTGNKQESNPSILNNNTNNNDNY 795
JMJD2A FGGFNQNC---G---NASDLAPQKQRTKPLIPEMCFTSTGCSTDINLSTP----YLEEDG 731
JMJD2B QTEKEAPIASLGKGCPATLPSKSRQKTRPLIPEMCFTSGGENTEPLPANS----YIGDDG 753
JMJD2C SSNEENDA---RWETKLDEVVTSEGKTKPLIPEMCFIYSEENIEYSPPNA----FLEEDG 711
zinc finger PHD type1
zinc finger C2H2 type
GIS1 RTSSMNNNGNNYQAHSSKFGENEVIMSDHGKIYICRECNRQFSSG---------HHLTRH 846
JMJD2A TSILVSCKKCSVRVHASCYGVPPAKAS---EDWMCSRCSANALEEDCCLCSLRGGALQRA 788
JMJD2B TSPLIACGKCCLQVHASCYGIRPELVN---EGWTCSRCAAHAWTAECCLCNLRGGALQMT 810
JMJD2C TSLLISCAKCCVRVHASCYGIPSHEIC---DGWLCARCKRNAWTAECCLCNLRGGALKQT 768
zinc finger PHD type1
zinc finger C2HC type
zinc finger C2H2 type
zinc finger C2H2 type
GIS1 --KKS----------VHS-----GEKPHSCPRCGKRFKRRDHVLQHLN 877
JMJD2A NDDRWVHVSCAVAILEARFVNIAERSPVDVSKIPLPRFKLKCIFCKKRRKRTAGCC---- 844
JMJD2B TDRRWIHVICAIAVPEARFLNVIERHPVDISAIPEQRWKLKCVYCRKRMKKVSGAC---- 866
JMJD2C KNNKWAHVMCAVAVPEVRFTNVPERTQIDVGRIPLQRLKLKCIFCRHRVKRVSGAC---- 824
zinc finger C2HC type
zinc finger PHD type2
GIS1 KKIPCTQEMENTKLAES---------- 894
JMJD2A --VQCSHGRCPTAFHVSCAQAAGVMMQPDDWPFVVFITCFRHKIPNLERAKGALQSITAG 902
JMJD2B --IQCSYEHCSTSFHVTCAHAAGVLMEPDDWPYVVSITCLKHKSGGH--AVQLLRAVSLG 922
JMJD2C --IQCSYGRCPASFHVTCAHAAGVLMEPDDWPYVVNITCFRHKVNPNVKSKACEKVISVG 882
zinc finger PHD type2
GIS1 ---------- 894
JMJD2A QKVISKHKNGRFYQCEVVRLTTETFYEVNFDDGSFSDNLYPEDIVSQDCLQFGPPAEGEV 962
JMJD2B QVVITKNRNGLYYRCRVIGAASQTCYEVNFDDGSYSDNLYPESITSRDCVQLGPPSEGEL 982
JMJD2C QTVITKHRNTRYYSCRVMAVTSQTFYEVMFDDGSFSRDTFPEDIVSRDCLKLGPPAEGEV 942

```
GIS1    ------------------------------------------------------------     894
JMJD2A  VQVRWTDGQVYGAKFVASHPIQMYQVEFEDGSQLVVKRDDVYTLDEELPKRVKSRLSVAS    1022
JMJD2B  VELRWTDGNLYKAKFISSVTSHIYQVEFEDGSQLTVKRGDIFTLEEELPKRVRSRLSLST    1042
JMJD2C  VQVKWPDGKLYGAKYFGSNIAHMYQVEFEDGSQIAMKREDIYTLDEELPKRVKARFSTAS    1002

GIS1    ------------------------------------------------------894
JMJD2A  DMRFNEIFTEKE-VKQEKKRQRVINSRYREDYIEPALYRAIME-------------1064
JMJD2B  GAPQEPAFSGEEAKAAKR--PRVGTPLATEDSGRSQDYVAFVESLLQVQGRPGAPF1096
JMJD2C  DMRFEDTFYGADIIQGERKRQRVLSSRFKNEYVADPVYRTFLKSSFQKKCQKRQ--1056
```

Figure 3.7. Sequence alignment of Gis1 with its mammalian orthologues JMJD2A/KDM4A, JMJD2B/KDM4B, and JMJD2C/KDM4C.

They have important functions in modulating histone methylation, hormonal signaling, and oxygen signaling. They are implicated in many disease processes including tumorigenesis, cardiac hypertrophy, and cardiomyopathy [3, 5, 6]. These mammalian JmjC domain-containing proteins have at least one CP motif and many other residues capable of chelating heme. Thus, they all have the potential to sense heme and change their activity in response to heme. Additionally, both KDM4A and Gis1 are associated with TOR signaling proteins and likely play important roles in nutrient signaling [68, 69]. As such, Gis1 may exemplify this new class of heme regulatory histone demethylases and transcriptional regulators. Understanding molecular mechanisms governing Gis1-mediated signaling is likely to facilitate our understanding of mechanisms governing KDM4 protein functions.

References

1. Klose, R.J. and Y. Zhang, *Regulation of histone methylation by demethylimination and demethylation.* Nat Rev Mol Cell Biol, 2007. **8**(4): p. 307-18.

2. Chen, Z., et al., *Structural insights into histone demethylation by JMJD2 family members.* Cell, 2006. **125**(4): p. 691-702.

3. Johansson, C., et al., *The roles of Jumonji-type oxygenases in human disease.* Epigenomics, 2014. **6**(1): p. 89-120.

4. Labbe, R.M., A. Holowatyj, and Z.Q. Yang, *Histone lysine demethylase (KDM) subfamily 4: structures, functions and therapeutic potential.* Am J Transl Res, 2014. **6**(1): p. 1-15.

5. Hoffmann, I., et al., *The role of histone demethylases in cancer therapy.* Mol Oncol, 2012. **6**(6): p. 683-703.

6. Hancock, R.L., et al., *Epigenetic regulation by histone demethylases in hypoxia.* Epigenomics, 2015. **7**(5): p. 791-811.

7. Zhang, L., *HEME BIOLOGY: The Secret Life of Heme in Regulating Diverse Biological Processes*. 2011: World Scientific.

8. Bock, K.W., F. De Matteis, and W.N. Aldridge, *Heme and Hemoproteins*. 1978, New York: Springer-Verlag.

9. Ponka, P., *Cell biology of heme.* Am J Med Sci, 1999. **318**(4): p. 241-56.

10. Ortiz de Montellano, P.R., *Hemes in Biology*, in *Wiley Encyclopedia of Chemical Biology*. 2009, John Wiley & Sons, Ltd: West Sussex. p. 240-249.

11. Barr, I., et al., *Ferric, not ferrous, heme activates RNA-binding protein DGCR8 for primary microRNA processing.* Proc Natl Acad Sci U S A, 2012. **109**(6): p. 1919-24.

12. Burton, M.J., et al., *A heme-binding domain controls regulation of ATP-dependent potassium channels.* Proc Natl Acad Sci U S A, 2016. **113**(14): p. 3785-90.

13. Ye, W.Z. and L. Zhang, *Heme controls the expression of cell cycle regulators and cell proliferation in HeLa Cells.* Biochem Biophys Res Commun, 2004. **315**: p. 546-554.

14. Zhu, Y., T. Hon, and L. Zhang, *Heme initiates changes in the expression of a wide array of genes during the early erythroid differentiation stage.* Biochem Biophys Res Commun, 1999. **258**: p. 87-93.

15. Hon, T., et al., *The yeast heme-responsive transcriptional activator Hap1 is a preexisting dimer in the absence of heme.* J Biol Chem, 1999. **274**: p. 22770-4.

16. Yao, X., et al., *Heme controls the regulation of protein tyrosine kinases Jak2 and Src.* Biochem Biophys Res Commun, 2010. **403**: p. 30-35.

17. Alam, M.M., et al., *A holistic view of cancer bioenergetics: mitochondrial function and respiration play fundamental roles in the development and progression of diverse tumors.* Clin Transl Med, 2016. **5**(1): p. 3.

18. Hooda, J., A. Shah, and L. Zhang, *Heme, an essential nutrient from dietary proteins, critically impacts diverse physiological and pathological processes.* Nutrients, 2014. **6**(3): p. 1080-102.

19. Klose, R.J., E.M. Kallin, and Y. Zhang, *JmjC-domain-containing proteins and histone demethylation.* Nat Rev Genet, 2006. **7**(9): p. 715-27.

20. Tu, S., et al., *Identification of histone demethylases in Saccharomyces cerevisiae.* J Biol Chem, 2007. **282**(19): p. 14262-71.

21. Kwon, D.W. and S.H. Ahn, *Role of yeast JmjC-domain containing histone demethylases in actively transcribed regions.* Biochem Biophys Res Commun, 2011. **410**(3): p. 614-9.

22. Melvin, A. and S. Rocha, *Chromatin as an oxygen sensor and active player in the hypoxia response.* Cell Signal, 2011. **24**(1): p. 35-43.

23. Boorstein, W.R. and E.A. Craig, *Regulation of a yeast HSP70 gene by a cAMP responsive transcriptional control element.* Embo J, 1990. **9**(8): p. 2543-53.

24. Pedruzzi, I., et al., *Saccharomyces cerevisiae Ras/cAMP pathway controls post-diauxic shift element-dependent transcription through the zinc finger protein Gis1.* Embo J, 2000. **19**(11): p. 2569-79.

25. Cameroni, E., et al., *The novel yeast PAS kinase Rim 15 orchestrates G0-associated antioxidant defense mechanisms.* Cell Cycle, 2004. **3**(4): p. 462-8.

26. Roosen, J., et al., *PKA and Sch9 control a molecular switch important for the proper adaptation to nutrient availability.* Mol Microbiol, 2005. **55**(3): p. 862-80.

27. Cheng, C., et al., *Inference of transcription modification in long-live yeast strains from their expression profiles.* BMC Genomics, 2007. **8**: p. 219.

28. Zhang, N., J. Wu, and S.G. Oliver, *Gis1 is required for transcriptional reprogramming of carbon metabolism and the stress response during transition into stationary phase in yeast.* Microbiology, 2009. **155**(Pt 5): p. 1690-8.

29. Wei, M., et al., *Tor1/Sch9-regulated carbon source substitution is as effective as calorie restriction in life span extension.* PLoS Genet, 2009. **5**(5): p. e1000467.

30. Orzechowski Westholm, J., et al., *Gis1 and Rph1 regulate glycerol and acetate metabolism in glucose depleted yeast cells.* PLoS One, 2012. **7**(2): p. e31577.

31. Jang, Y.K., L. Wang, and G.B. Sancar, *RPH1 and GIS1 are damage-responsive repressors of PHR1*. Mol Cell Biol, 1999. **19**(11): p. 7630-8.

32. Sein, H., S. Varv, and A. Kristjuhan, *Distribution and maintenance of histone H3 lysine 36 trimethylation in transcribed locus.* PLoS One, 2015. **10**(3): p. e0120200.

33. Yu, Y., A.M. Neiman, and R. Sternglanz, *The JmjC domain of Gis1 is dispensable for transcriptional activation.* FEMS Yeast Res, 2010. **10**(7): p. 793-801.

34. Quan, Z., S.G. Oliver, and N. Zhang, *JmjN interacts with JmjC to ensure selective proteolysis of Gis1 by the proteasome.* Microbiology, 2011. **157**(Pt 9): p. 2694-701.

35. Zhang, L. and L. Guarente, *Heme binds to a short sequence that serves a regulatory function in diverse proteins.* Embo J, 1995. **14**(2): p. 313-20.

36. Kuhl, T., et al., *Analysis of Fe(III) heme binding to cysteine-containing heme-regulatory motifs in proteins.* ACS Chem Biol, 2013. **8**(8): p. 1785-93.

37. Suzuki, H., et al., *Heme regulates gene expression by triggering Crm1-dependent nuclear export of Bach1*. Embo J, 2004. **23**(13): p. 2544-53.

38. Hira, S., et al., *Bach1, a heme-dependent transcription factor, reveals presence of multiple heme binding sites with distinct coordination structure.* IUBMB Life, 2007. **59**(8-9): p. 542-51.

39. Hon, T., et al., *Functional analysis of heme regulatory elements of the transcriptional activator Hap1*. Biochem Biophys Res Commun, 2000. **273**: p. 584-591.

40. Lee, H.C., et al., *Structural environment dictates the biological significance of heme-responsive motifs and the role of Hsp90 in the activation of the heme activator protein Hap1.* Mol Cell Biol, 2003. **23**(16): p. 5857-66.

41. Henke, R.M., et al., *Hypoxia elicits broad and systematic changes in protein subcellular localization.* Am J Physiol Cell Physiol, 2011. **301**(4): p. C913-28.

42. Dastidar, R.G., et al., *The nuclear localization of SWI/SNF proteins is subjected to oxygen regulation.* Cell Biosci, 2012. **2**(1): p. 30.

43. Zhang, L. and A. Hach, *Molecular mechanism of heme signaling in yeast: The transcriptional activator Hap1 serves as the key mediator.* Cell Mol Life Sci, 1999. **56**: p. 415-426.

44. Haldi, M. and L. Guarente, *N-terminal deletions of a mitochondrial signal sequence in yeast. Targeting information of delta-aminolevulinate synthase is encoded in non-overlapping regions.* J Biol Chem, 1989. **264**(29): p. 17107-12.

45. Zhang, N. and S.G. Oliver, *The transcription activity of Gis1 is negatively modulated by proteasome-mediated limited proteolysis.* J Biol Chem, 2010. **285**(9): p. 6465-76.

46. Turcotte, B. and L. Guarente, *HAP1 positive control mutants specific for one of two binding sites.* Genes Dev, 1992. **6**(10): p. 2001-9.

47. Zhang, L. and L. Guarente, *The C6 zinc cluster dictates asymmetric binding by HAP1.* Embo J, 1996. **15**(17): p. 4676-81.

48. Hon, T., H.C. Lee, and L. Zhang, *The actions of Hsp90 and Hsp70 molecular chaperones in heme signaling in eukaryotes*, in *Recent Research Developments in Molecular and Cellular Biology*, J. Fagan, N. Shimizu, and J.N. Davidson, Editors. 2003, Research Signpost: Trivandrum, India. p. 11-21.

49. Kundaje, A., et al., *A predictive model of the oxygen and heme regulatory network in yeast.* PLoS Comput Biol, 2008. **4**(11): p. e1000224.

50. Guarente, L. and T. Mason, *Heme regulates transcription of the CYC1 gene of S. cerevisiae via an upstream activation site.* Cell, 1983. **32**(4): p. 1279-86.

51. Hon, T., et al., *The heme activator protein Hap1 represses transcription by a heme-independent mechanism in Saccharomyces cerevisiae.* Genetics, 2005. **169**(3): p. 1343-52.

52. Pringle, J.R., et al., *Immunofluorescence methods for yeast.* Methods Enzymol, 1991. **194**: p. 565-602.

53. Pringle, J.R., et al., *Fluorescence microscopy methods for yeast.* Methods Cell Biol, 1989. **31**: p. 357-435.

54. Hon, T., et al., *The Hsp70-Ydj1 Molecular Chaperone Represses the Activity of the Transcriptional Activator Hap1 in the Absence of Heme.* Mol Cell Biol, 2001. **21**: p. 7923-7932.

55. Oshiro, J., et al., *Regulation of the yeast DPP1-encoded diacylglycerol pyrophosphate phosphatase by transcription factor Gis1p.* J Biol Chem, 2003. **278**(34): p. 31495-503.

56. Huh, W.K., et al., *Global analysis of protein localization in budding yeast.* Nature, 2003. **425**(6959): p. 686-91.

57. Ansari, A.Z., et al., *Towards a minimal motif for artificial transcriptional activators.* Chem Biol, 2001. **8**(6): p. 583-92.

58. Hach, A., T. Hon, and L. Zhang, *A new class of repression modules is critical for heme regulation of the yeast transcriptional activator Hap1.* Mol Cell Biol, 1999. **19**: p. 4324-4333.

59. Balciunas, D. and H. Ronne, *Yeast genes GIS1-4: multicopy suppressors of the Gal- phenotype of snf1 mig1 srb8/10/11 cells.* Mol Gen Genet, 1999. **262**(4-5): p. 589-99.

60. Galdieri, L., et al., *Transcriptional regulation in yeast during diauxic shift and stationary phase.* Omics, 2010. **14**(6): p. 629-38.

61. Iuchi, S., *Three classes of C2H2 zinc finger proteins.* Cell Mol Life Sci, 2001. **58**(4): p. 625-35.

62. Brown, R.S., *Zinc finger proteins: getting a grip on RNA.* Curr Opin Struct Biol, 2005. **15**(1): p. 94-8.

63. Gamsjaeger, R., et al., *Sticky fingers: zinc-fingers as protein-recognition motifs.* Trends Biochem Sci, 2007. **32**(2): p. 63-70.

64. Liew, C.K., et al., *Zinc fingers as protein recognition motifs: structural basis for the GATA-1/friend of GATA interaction.* Proc Natl Acad Sci U S A, 2005. **102**(3): p. 583-8.

65. Razin, S.V., et al., *Cys2His2 zinc finger protein family: classification, functions, and major members.* Biochemistry (Mosc), 2012. **77**(3): p. 217-26.

66. Loughlin, F.E. and J.P. Mackay, *Zinc fingers are known as domains for binding DNA and RNA. Do they also mediate protein-protein interactions?* IUBMB Life, 2006. **58**(12): p. 731-3.

67. Berry, W.L. and R. Janknecht, *KDM4/JMJD2 histone demethylases: epigenetic regulators in cancer cells.* Cancer Res, 2013. **73**(10): p. 2936-42.

68. Carbonneau, M., et al., *The oncometabolite 2-hydroxyglutarate activates the mTOR signalling pathway.* Nat Commun, 2016. **7**: p. 12700.

69. Collins, S.R., et al., *Functional dissection of protein complexes involved in yeast chromosome biology using a genetic interaction map.* Nature, 2007. **446**(7137): p. 806-10.

CHAPTER 4

DISCUSSION AND CONCLUSION

4.1 Discussion

Oxygen is an important signaling molecule for aerobes. To cope with changing oxygen levels, yeast and higher eukaryotes have developed sophisticated mechanisms at the level of protein localization and translational modifications. Genome-wide changes in the protein localization under hypoxia in yeast was first studied by Henke *et. al.* [1]. They found that over 200 proteins are redistributed under hypoxia. Gene network study of these redistributed proteins showed that they belong to important regulatory complexes. Of the 200 proteins, several proteins of the SWI/SNF complex was found to be redistributed under hypoxia in a comparatively short time. Interestingly, the target genes of the SWI/SNF complex are also found to be regulated by oxygen [2], which shows that SWI/SNF complex plays important function in oxygen regulation and aerobic respiration. Further experiments were carried out to understand the role of the SWI/SNF complex proteins in regulating aerobic respiration. The results show that Swi3 plays an important role in moderating respiration and oxygen consumption. Also, the mitochondrial respiratory chain complex proteins were found to be upregulated in cells with knockout of *SWI3.* Computational analysis of the target genes of Swi3 human homologues BAF155 and BAF170 provide evidence that they belong to the oxidative phosphorylation process. The rate of oxygen consumption was increased in the knock down cells of BAF155 and BAF170. These results clearly provide substantiation that Swi3 and its human homologues are important players in regulating aerobic respiration.

Microarray gene expression studies have shown that more than 300 genes are induced by heme and oxygen both in yeast Saccharomyces *cerevisiae* [3], of which Hap1 regulates the activation of 27 genes, and suppresses 21 genes. Thus, there are numerous genes still to be identified and studied whose activity is mediated by heme and oxygen. A JumonjiC (JmjC) domain containing transcription factor Gis1, is widely studied as a stress-response transcription factor. In our lab, we identified it as a fast responder to change in oxygen levels. Under hypoxia, Gis1 which is a nuclear protein is retained in the cytoplasm [1]. Gis1 protein has two CP motifs, one in its JmjC domain and the other one in its second ZnF domain. Hence, we performed experiments to study if Gis1 is regulated by heme. We found that the transcriptional activity of Gis1 is indeed induced under high heme, although heme does not affect its binding to DNA. The JmjN+JmjC domain of Gis1 is dispensable for its heme activation of transcriptional activity. Using the fusion construct of various fragments of Gis1 with the DNA binding domain of an unrelated protein showed that the ZnF domain of Gis1 is responsible for the heme regulation of Gis1. The JmjN+JmjC domain can induce transcriptional activity in an unrelated protein under high heme. In summary, we show that the transcriptional activity of Gis1 is mediated by heme, although heme does not affect the binding of Gis1 to the DNA.

4.2 Conclusion

The first half of the dissertation helps us understand how SWI/SNF complex is involved in oxygen sensing and regulation of aerobic respiration. Swi3 suppresses the aerobic respiration genes under high heme conditions. Also, the mitochondrial complex proteins are increased in the absence of Swi3. This shows that Swi3 regulates aerobic respiration. The second half of the dissertation involves studying regulation of JmjC domain containing protein called Gis1 by heme

levels. We show that Gis1 belonging to the JMJD2/KDM4 family of proteins, is a unique transcription factor whose transcriptional activity is regulated by heme.

References

1. Henke, R.M., et al., *Hypoxia elicits broad and systematic changes in protein subcellular localization.* Am J Physiol Cell Physiol, 2011. **301**(4): p. C913-28.

2. Dastidar, R.G., et al., *The nuclear localization of SWI/SNF proteins is subjected to oxygen regulation.* Cell Biosci, 2012. **2**(1): p. 30.

3. Zhang, L., *HEME BIOLOGY: The Secret Life of Heme in Regulating Diverse Biological Processes*. 2011: World Scientific.

4. Tu, S., et al., *Identification of histone demethylases in Saccharomyces cerevisiae.* J Biol Chem, 2007. **282**(19): p. 14262-71.

BIOGRAPHICAL SKETCH

Sneha Lal was born in Chennai, Tamil Nadu, India. She attended Jawahar Vidyalaya Senior Secondary School, Chennai, India till the 6th grade. She then moved to Vadodara, Gujarat, India where she completed schooling from Kendriya Vidyalaya, ONGC, Vadodara, India. She graduated with a Bachelor of Science (BSc) degree in Biotechnology from Sardar Patel University, Gujarat, India in 2008. From 2008-2010 she pursued a Master of Science degree (MSc) in Bioinformatics from Maharaja Sayajirao University of Baroda, Gujarat, India. In 2011, she joined the Department of Biological Sciences at The University of Texas at Dallas to pursue her doctoral degree.

CURRICULUM VITAE

SNEHA LAL

sneha.lal@utdallas.edu

EDUCATION

University of Texas at Dallas, Dallas
Dallas, TX, USA
PhD (Molecular and Cell Biology) 2011-2017

Maharaja Sayajirao University of Baroda, Gujarat
Gujarat, India
MSc (Bioinformatics) 2008-2010

Sardar Patel University, Gujarat
Gujarat, India
BSc (Biotechnology) 2005-2008

RESEARCH EXPERIENCE

University of Texas at Dallas, Dallas Dallas, TX, USA
Graduate student; Advisor: Prof. Li Zhang 2011-present
'Understanding heme regulation of transcriptional activities of JmjC domain containing transcription factor Gis1 in *S. cerevisiae'*

- Examined the localization of Gis1 under air, hypoxia, low and high amounts of heme
- Examined the effect of heme on the transcriptional and demethylase activities of various fragments of Gis1
- Purified Gis1 and studied its heme binding
- Constructed fusion proteins of various fragments of Gis1 and a heterogenous DNA binding domain to study the transcriptional regulation of Gis1

'The Swi3 protein plays a unique role in regulating respiration in eukaryotes'

- Examined the rate of oxygen consumption of the knockout strains of SWI/SNF component proteins
- Isolated mitochondria, analyzed mitochondrial chain respiratory chain complexes in the *Δswi2* and *Δswi3* strains
- Examined the effect of deleting SWI2 or SWI3 on the promoter activities of respiration genes

M.S. University of Baroda, Gujarat Gujarat, India
Graduate student; Advisor: Dr. Prashanth Murumkar 2010
Pharmacophore generation of 11β- HSD1 inhibitors and homology modeling of 11β- HSD2

- Prepared a dataset of the reported structures of 11β-HSD1 inhibitors using SYBYL 7.0 software running on Silicon Graphics Fuel workstation
- Generated a pharmacophore model using this dataset in PHASE
- Developed the structure of 11β-HSD2 by homology modeling using the software MODELLER v.2.0 and analyzed the protein model by Ramachandran Plot

SKILLS

- DNA, RNA, Protein and Mitochondria isolation, antibody purification
- Bacterial and yeast transformation, expression and purification of proteins from bacteria
- Gene cloning, gene disruption and gene replacement in yeast, genetic screens in yeast
- Plasmid construction, PCR, qPCR
- Immuno fluorescence, Fluorescence microscopy
- Chromatin immunoprecipitation (ChIP), enzyme assays, electrophoretic mobility shift assays (EMSA)
- Heme synthesis, heme uptake and oxygen consumption assays
- Western blot, Blue-native PAGE
- Mammalian cell culture

TEACHING EXPERIENCE

- **University of Texas at Dallas, Dallas** Dallas, TX, USA
- Head Teaching assistant, Biochemistry Spring 2013, Fall 2013
- Teaching assistant, Biochemistry Fall 2011, Spring 2012, Fall 2012, Spring 2017
- Teaching assistant, Intro Bio Lab Spring 2014
- Teaching assistant, Introduction to Modern Biology Summer 2012, Summer 2013
- Teaching assistant, Molecular Biology of Cancer/Oncogenes Summer 2017

- **N. V. Patel College of Pure and Applied Sciences, Anand** Gujarat, India
- Appointed as Lecturer, Dept. of Biosciences January 2011- April 2011

PUBLICATIONS

Lal S, Comer JM, Konduri PC, Shah A, Wang T, Lewis A, Shoffner G, Guo F, Zhang L (2017) Heme promotes transcriptional and demethylase activities of the JmjC domain-containing protein Gis1. Nucleic Acids Research, gkx1051

Lal S, Alam MM, Hooda J, Shah A, Cao TM, Xuan Z, Zhang L (2016) The Swi3 protein plays a unique role in regulating respiration in eukaryotes. Bioscience reports 36

Alam MM, **Lal S**, FitzGerald KE, Zhang L (2016) A holistic view of cancer bioenergetics: mitochondrial function and respiration play fundamental roles in the development and progression of diverse tumors. Clinical and translational medicine 5: 3

FitzGerald KE, **Lal S**, Kalainayakan SP and Zhang L (2016) Molecular mechanisms underlying heme action in promoting the pathogenesis of Alzheimer's Disease. http://www.smgebooks.com/alzheimers-disease/chapters/ALZD-16-04.pdf

PROFESSIONAL SERVICES

- Organized the 25th Royston Clowes Memorial Lecture Series 2014, UT Dallas, invited Dr. Jeffrey Zigman from UT Southwestern Medical Center, Dallas, TX
- Organized the 26th Royston Clowes Memorial Lecture Series 2015, UT Dallas, invited Dr. Timothy Donohue from University of Wisconsin-Madison, Wisconsin

SEMINARS AND CONFERENCES ATTENDED

- Poster presentation at Yeast Genetics Meeting 2014: 'Swi3, a novel regulator of aerobic respiration genes and oxygen metabolism in Saccharomyces cerevisiae'. Abstract: 205A
- Poster presentation at 28th Fungal Genetics Conference 2015: 'Novel function of Swi3 in moderating aerobic respiration and oxygen consumption in Saccharomyces cerevisiae'. Abstract: 408
- Poster presentation at The Allied Genetics Conference 2016: 'Understanding heme regulation of JmjC domain containing transcription factor Gis1'. Abstract: Y3114C
- Attended the National Symposium on Exploring New Horizons in Microbial Biotechnology Colloquim-2011, Depts of Microbiology, Biotechnology, Genetics and Bioinformatics, N. V. Patel College of Pure and Applied Sciences (NVPAS), Anand, India.
- Attended the 1st International Federation of Information Processing (IFIP) International Conference on Bioinformatics, Department of Applied Mathematics and Humanities, Sardar Vallabhbhai National Institute of Technology, Surat, India.
- Attended the National Conference, 'CME in Immunology', 2009, organized by Department of Biochemistry, M. S. University of Baroda & Indian Immunology Society, India.
- Pursued training in 'Basic Bioinformatics Techniques using Tools for Biological Databases' for a month from Centre for Genome Research, M. S. University of Baroda, Vadodara, India.
- Attended a state level seminar on 'New Horizons in Biological Sciences' jointly organized by NVPAS, Vallabh Vidyanagar and Gujarat State Biotechnology Mission (GSBTM), Gandhinagar, India.