THE PAQR FAMILY OF MEMBRANE PROTEINS IN *Saccharomyces cerevisiae*: THEIR ROLE IN SIGNAL TRANSDUCTION AND FLOW CYTOMETRIC ANALYSIS OF AdipoR1 BINDING TO ADIPONECTIN AND TNF-ALPHA

By

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To my family, whose faith and strength are my driving force to deal with adversity, and the challenges in my life.

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THE PAQR FAMILY OF MEMBRANE PROTEINS IN
Saccharomyces cerevisiae: THEIR ROLE IN THE SIGNAL TRANSDUCTION PATHWAY
OF *FET3* AND FLOW CYTOMETRIC ANALYSIS OF AdipoR1 BINDING TO
ADIPONECTIN AND TNF-ALPHA

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Major: Chemistry

The yeast *Saccharomyces cerevisiae* contains a family of zinc responsive membrane proteins named *IZH1-4* (Implicated in Zinc Homeostasis), which belong to a superfamily of proteins known as the PAQRs (Progestin AdipoQ Receptor). These putative membrane proteins contain at least seven transmembrane domains. The PAQR family of proteins consists of known osmotin, adiponectin and progesterone receptors that are distantly related to alkaline ceramidases. The objective of this study is to characterize the role of the PAQR family in lipid mediating signaling. We have evidence that the PAQR family, when overexpressed or in the presence of their specific ligand, represses *ZRT1*, a high affinity zinc transporter gene, and *FET3*, a high affinity iron transporter gene, suggesting a role for these proteins in zinc- and iron-uptake. The repression of these genes requires a sphingoid-base dependent signal transduction pathway involving Ras-cAMP/PKA and the Snf1p AMP-dependent kinase. This pathway ultimately governs a competition between the Nrg1p/Nrg2p and Msn2p/Msn4p transcription factors for binding sites in the promoter region of *ZRT1* and *FET3*.

We also found that human AdipoR1 represses *FET3* with the addition of adiponectin. Furthermore, the addition of TNF-α partially alleviates repression by AdipoR1. However, when

adiponectin and TNF-α were added together, *FET3* repression was not alleviated. Finally, we examined labeled-adiponectin and TNF-α binding to cells overexpresing AdipoR1. Bound TNF-α levels fell when unlabeled adiponectin was added; however, the addition of unlabeled TNF-α displaced labeled-adiponectin. Thus, adiponectin and TNF-α compete for binding to yeast cells expressing AdipoR1.

CHAPTER 1
INTRODUCTION

Iron and Zinc Homeostasis

Zinc and iron are essential nutrients for all organisms and play important roles in many biochemical processes. Zinc, a strong lewis acid, is relatively inert. It is an essential component of over 300 enzymes, such as Cu/Zn dismutase and alcohol dehydrogenases, and plays a critical structural role for many proteins (Vallee and Auld, 1999; Eide, 1998). Iron, unlike zinc, can donate and accept electrons and is an important cofactor in electron transport and many redox-active metalloenzymes. Since iron and zinc are the most abundant trace elements in human cells (Lyons et al., 2000), a deficiency or excess of these metals could lead to a variety of problems. Severe zinc deficiency is linked to rheumatoid arthritis, central nervous system disorders, acodermatitis enteropathica, and impaired growth and immune function (Brown et al., 1998) (Beyersman and Harase, 2001). Meanwhile, zinc toxicity interferes with mitochondrial aconitase activity, thereby affecting cellular respiration (Costello et al., 1997). Iron deficiency can lead to anemia, but an excess, is linked to hemochromatosis, which can lead to organ failure and chronic diseases such as cirrhosis and diabetes (Dallman et al., 1980). Because of these considerations, organisms have evolved regulatory systems to control uptake, distribution, and detoxification of metal ions. Most of these regulatory systems were first identified in the budding yeast, *Saccharomyces cerevisiae*, thus making it an excellent model system; first, metal transport has been extensively studied in this organism; second, *S. cerevisiae* has been used to identify genes involved in metal transport in higher eukaryotes, including humans (Ho et al., 2002).

Zinc uptake in *S. cerevisiae* is controlled in response to zinc levels inside the cell. During zinc limitation, *ZRT1*, the high affinity zinc transporter, is induced (Zhao and Eide, 1996). Similarly low affinity zinc transporter is induced by low zinc levels and controlled by *ZRT2*

transcription. Regulation of these genes is mediated by the *ZAP1* (**Z**inc-responsive **A**ctivator **P**rotein) gene (Figure 1-1), which encodes a transcriptional activator with seven carboxy-terminal C_2H_2 zinc finger domains and two amino-terminal activation domains (Zhao and Eide 1997). Zap1p binds to a ZRE (**Z**inc **R**esponse **E**lement) consensus sequence, 5'-ACCTTNAAGGT, in the promoters of *ZRT1*, *ZRT2*, *ZRC1* (**Z**inc **R**esistance **C**onferring, vacuolar membrane storage transporter), *FET4* (low affinity zinc transporter) and *ZAP1* itself (Figure 1-2) (Zhao et al.,1998; Lyons et al., 2000).

The availability of iron in the cell affects the regulation of iron uptake in *S. cerevisiae*, which is sensed by the transcription factor Aft1p. Aft1p is known to bind to a consensus sequence known as the FeRE (**Fe** **R**esponse **E**lement) (Figure 1-3), 5'-TGCACCC, in the promoter region of a variety of genes. During iron limitation, many genes are induced, including, *FRE1* (**F**erric **RE**ductase), *FRE2*, *FET3* (**FE**rrous **T**ransport), and *FTR1* (**F**e **TR**ansporter) (Babcock et al., 1997; Dancis et al., 1992). Unlike the zinc uptake system, the high-affinity iron uptake system is highly specific for Fe^{3+}. This specificity is conferred by multiple chemical reactions in which Fe^{3+} is first reduced by Fre1p or Fre2p, then handed to Fet3p that oxidizes Fe^{2+} to Fe^{3+}. Finally Ftr1p, which is directly coupled to Fet3p, allows Fe^{3+} to enter the cell (Askith and Kaplan, 1997). Figure 1-4 shows the membrane transport proteins involved in the acquisition of iron in yeast.

Identification of the *IZH*s

In order to define the Zap1p regulon and identify genes involved in zinc metabolism, DNA microarrays and computer analysis of shared motifs in the promoters of co-regulated genes were performed by Lyons, *et al* (Lyons et al., 2000). These analyses identified 46 genes regulated by Zap1p. Two of these genes identified, Y*DR492w* and *YOL002c*, encode

homologous proteins that are induced by zinc deficiency in a Zap1p dependent manner. This was due to the presence of a ZRE found in the promoter region of *YDR492w* and *YOL002c*. A third protein, *YOL101c*, was identified in a separate microarray experiment to be induced by excess zinc (3 mM). Also in this high zinc experiment *YOL002c* was induced. An additional gene, *YLR023c*, related to *YDR492w*, *YOL002c*, and *YOL101c* was identified by BLAST and PSI-BLAST searches. Since three of these four genes in yeast respond to changes in zinc levels inside the yeast cell, they were renamed *IZH*s (**I**mplicated in **Z**inc **H**omeostasis): *YDR492w* (*IZH1*), *YOL002c* (*IZH2*), *YLR023c* (*IZH3*), and *YOL101c* (*IZH4*).

The *IZH*s have been implicated in other roles independent of Zap1p regulation. First, *IZH4* was found to respond to excess Zn^{2+}, Co^{2+}, and Ni^{2+} or deficiency in Fe^{3+} limitation by Mga2p, a transcription factor that regulates hypoxia. Second, *IZH2* is regulated by the fatty acid, mystiric acid (Lyons et al., 2004). Last, they have seen to be also involved in the regulation of genes involved in iron-uptake (Kupchak et al., 2007).

Structural Features

The *IZH* genes encode proteins that belong to a large family of membrane proteins called PAQR (Progestin and AdipoQ Receptors), which consist of four yeast and eleven human proteins. Alignments of these proteins in figures 1-5 and 1-6 demonstrate that these proteins can be classified into three distinct classes:

1) Class I: Four yeast proteins: Izh1p, Izh2p, Izh3p, and Izh4p

   Four human proteins: AdipoR1 (**Adipo**nectin **R**eceptor), AdipoR2, PAQR3, and PAQR4

2) Class II: mPRγ (**m**embrane **P**rogestin **R**eceptor), PAQR6, mPRα, mPRβ, and PAQR9

3) Class III: PAQR10 and PAQR11

Further investigation of protein sequences showed the PAQR proteins within each class exhibited close sequence similarity. In Class I, the yeast Izh1p and Izh2p share 39% amino acid identity, while the human PAQRs, AdipoR1 and AdipoR2 exhibit 68% identity. Sequence similarity was also seen in Class II, where mPRα can be paired with mPRβ and mPRγ with PAQR6. Figure 1-6 also showed Class III PAQR10 and PAQR11 were very similar.

The PAQR protein family consists of at least seven transmembrane (TM) domains (Figure 1-7), but lacks significant amino acid similarity. When the PAQR family of proteins is aligned, they exhibit only three highly conserved regions located on the intracellular side of the membrane. The first motif is located at the start of the TM1, $Ex_{2\text{-}3}Nx_3H$, but can be expanded to $Px_nGx_nS/Tx_nEx_{2\text{-}3}Nx_3H$ if the Class I and Class II PAQRs are considered. Motif two consists of an Sx_3H at the end of TM2 and an aspartic acid at the beginning of TM3. Motif 3 has an Hx_3H sequence in the loop region between TM6 and TM7, which can be expanded to PGxnPExnHx3H if only Class I and Class II are considered. Also, hydropathy plots demonstrate that Class II PAQR proteins contain an additional region of hydrophobic amino acids, which can be thought of as an eighth TM domain (Figure 1-8).

Even though the topology of the PAQR proteins is uncertain, it has been suggested that the PAQR proteins all have an internal N-terminus and external C-terminus, except for the Class II PAQR proteins, which would have an internal C-terminus. However, the only assessment that could be confirmed at this time is that two human adiponectin receptors (AdipoR1 and AdipoR2) (Yamauchi et al., 2003) and yeast Izh2p (Kim et al., 2006) and Izh4p (Kim et al., 2003) have an external C-terminus. Based on structural prediction programs, the Izh proteins have been suggested to be localized to the plasma membrane, while the human PAQRs localization remains in question, either residing in the plasma membrane or endoplasmic reticulum. The conserved

motifs found on the intracellular loops may indicate the PAQR proteins all signal in a similar manner. The extracellular loop regions exhibit no homology, but this may explain why the Izh and human PAQR proteins bind to three different ligands: osmotin, adiponectin, and progesterone. Considering that the conserved regions are intracellular, we propose that yeast *IZH*s or human PAQRs exhibit a conservation of signaling mechanism.

The mPRs have often been confused with the G-protein coupled receptors (GPCRs) based on their topology and mechanism of signal transduction. First, they both are predicted to possess seven TM domains with an extracellular N-terminus and an intracellular C-terminus. Second, pertussis toxin, an inhibitor of $G_{i\alpha}$, also inhibits the effects of mPRs (Zhu et al., 2003a). However, these characteristics do not prove the PAQRs are GPCRs. To refute these claims, $G_{i\alpha}$ involvement in GPCR-mediated signaling would have to be direct, but this may not be the case for the mPRs. Also the PAQRs and GPCRs share no sequence homology beyond the presence of seven predicted TM domains (Lyons et al., 2004); however, the topology and number of TM domains remains unconfirmed, since no groups have mapped the PAQR protein topology. Last, the topology of the GPCRs appears to opposite side to that of AdipoR1 and AdipoR2 (Yamauchi et al., 2003). Thus the similarities between the PAQR and GPCR proteins are only speculative, but this does not rule out the PAQRs are distantly related to GPCRs.

Class I, II, and III Receptors

The yeast Izh and human PAQR receptors can be classified into three categories based on sequence similarities. The human adiponectin receptors, AdipoR1 (PAQR1) and AdipoR2 (PAQR2), and the osmotin receptors, OsmoR (Izh2p), found in various species of yeast make up the class I receptors. There also exists a similarity between the human adiponectin receptors and the yeast Izh1p and Izh2p. However, Izh3p and Izh4p along with PAQR3 and PAQR4 are more

divergent and distantly related but still can be considered to be part of this class. Most of the class I proteins can be found throughout metazoan evolution ranging from fungi to humans.

One of these receptors, Izh2p, was recently discovered to bind to osmotin (Narasimhan et al., 2001). Osmotin is a 24 kDa protein that belongs to family known as PR-5 defensins. Moreover, osmotin was first identified in tobacco plants and exhibits antifungal properties against the pathogenic *Candida albicans* and the non-pathogenic *Sacchromyces cerevisiae* (Koiwa et al., 1997). Osmotin is not only induced by desiccation and salinity, but also by abscissic acid, wounding and cold temperatures (Zhu et al., 1993; Zhu et al., 1995). Osmotin is suggested to be involved in a pathway that requires the Ras2p G protein, which ultimately may lead to apoptosis of the yeast cell (Narasimhan et al., 2005). Early theories postulated that osmotin may induce holes in the fungal wall though a specific interaction with the plasma membrane (De Vos et al., 1985) (Roberts and Selitrennikoff, 1986). However, the mechanism by which osmotin starts a signaling cascade that leads to plasma membrane permeabilization remains unknown.

The human Class I PAQRs, AdipoR1 and AdipoR2, bind to the adipose secreted ligand known as adiponectin (Yamauchi et al., 2003). Adiponectin is a 244 amino acid protein of approximately 30 kDa that is encoded by the adiponectin gene, apM1, located on chromosome 3q27. Also called gelatin-binding protein-28 (GBP28), ACRP30 or AdipoQ, adiponectin belongs to the complement C1q protein family (Scherer et al., 1995) to which the inflammatory tumor necrosis factor-α belongs. Originally, adiponectin was thought to be expressed and secreted only by adipocytes (Hu et al., 1996), but recent studies have detected adiponectin in skeletal muscle and liver cells (Yamauchi et al., 2003; Delaigle et al., 2004).

Adiponectin is one of the most abundant proteins found in human plasma, with concentrations ranging from 3 to 30 μM (Chandran et al., 2003). Unlike many adipose derived proteins, levels of adiponectin are found to be lower in obese than in lean individuals. Studies have shown that low levels of adiponectin are associated with diseases such as obesity, insulin resistance, and type 2 diabetes, cardiovascular disease and hypertension (Trijillo and Scherer; 2005). Additionally, adiponectin expression is down regulated by insulin and recent studies suggest adiponectin levels may be affected by dietary factors (Qi et al., 2005). In this case, it has been shown that calorie restriction and low glycemic diets can raise adiponectin levels (Qi et al., 2005). Low levels of adiponectin have also been reported to be associated with certain types of malignancies, such as endometrial cancer (Petridou, et al., 2003), breast cancer (Mantzoros et al., 2004), leukemia (Petridou et al., 2006), colon caner (Ishikawa et al., 2005), and prostate cancer (Goktas et al., 2005).

Adiponectin consists of four domains, including a signal peptide at the N-terminus, short variable region, a collagen-like motif and a C-terminus globular domain. Additionally, adiponectin is known to circulate in two forms. First, the low molecular weight (LMW, globular) oligimer consists of two trimers and second, the high molecular weight (HMW, full length) oligimer is made of four to six trimers, which is the predominant form in human plasma (Pajvani et al., 2004) (Figure 1-9). The two forms of adiponectin possess distinct signaling activities. Both the HMW and LMW forms induce NF-κB activation, whereas the LMW form induces AMP-protein kinase (AMPK) activation in muscle (Tsao et al., 2002). The activation of AMPK increases glucose uptake (Kurth-Kraczek et al., 1999), glycogen formation (Merrill et al., 1997), and inhibition of acetyl-CoA carboxylase (ACC), which leads to decreased fatty acid synthesis (Tomas et al., 2002). Adiponectin may also have additional roles in controlling

inflammation and having anti-atherogenic properties thought to be caused by monocytes adhering to the blood vessel walls (Ouchi et al., 1999).

AdipoR1 and AdipoR2 are derived from different genes located on chromosomes 1q23 and 12p13 that encode 376 and 386 amino acid polypeptides, respectively. Each receptor has been shown to have different distributions and affinities for the two forms of adiponectin. Also, AdipoR1 and AdipoR2 exhibit significant similarity in showing 67% amino acid identity (Yamauchi et al., 2003). AdipoR1 shows a high affinity for globular adiponectin, but can also bind to full length adiponectin at a lower affinity, and is expressed mainly in skeletal muscle; however, it is also found in liver and endothelial cells (Goldstein and Scalia; 2004). Meanwhile, AdipoR2 can be found at high levels in the liver and exhibits moderate affinity for globular and full length adiponectin (Goldstein and Scalia; 2004).

The Class II receptors, a new class of membrane progestin receptors (mPR) that mediate rapid, non-genomic steroid actions, were identified in spotted seatrout ovaries, and shown to mediate the induction of oocyte maturation (Zhu et al., 2003a). Furthermore, the Class II receptors are presumed to contain a hypothetical eighth transmembrane domain, which is a major difference between the Class I and Class III proteins. These receptors are known as mPRα (PAQR7), mPRβ (PAQR8), mPRγ (PAQR5) and bind to progesterone (Zhu et al., 2003b). Additionally, Class II also includes the proteins PAQR6 and PAQR9.

Progesterone is a C-21 steroid hormone that is required for many aspects of female reproduction, including sexual behavior, gonadotrophin secretion, implantation of blastocyst, and maintenance of pregnancy (Mulac-Jericevic et al., 2000) (Conneely et al., 2002). This hormone is produced in the adrenal glands, ovaries, corpus luteum after ovulation, the brain, and in the

placenta during pregnancy. Progesterone also has therapeutic applications ranging from birth control to the treatment of endometrial cancers (Sitruk-Ware and Plu-Bureau, 1999).

Progesterone, like all other steroids, is synthesized from pregnenolone, a derivative of cholesterol. The conversion of pregnonolone to progesterone takes place in two steps. The 3-hydroxyl group is converted to a keto group and a double bond is moved from C-5 to C-4. Also, progesterone is the precursor of the mineralocorticoid aldosterone and after conversion to 17α-hydroxyprogesterone can be synthesized into cortisol and androstenedione. Androstenedione can be further converted to testosterone and estradiol (Figure 1-10).

Many of the effects of progesterone occur through nuclear progesterone receptors; however, it has been known for the last 30 years that some of the effects occur too rapidly to be involved in the "classical signaling pathway" (Edwards, 2005). This type of signal transduction involves the steroid entering the target cell by passive diffusion through the plasma membrane, passing through the nuclear membrane and binding to a nuclear receptor. The steroid-receptor complex then undergoes a conformational change and binds to DNA sequences known as steroid response elements. There the steroid-receptor acts as a transcription factor modulating gene transcription and synthesis of mRNAs and proteins. However, there are examples where the action of steroids cannot be explained by the classical signaling model. This signaling occurs far too rapidly to require gene transcription and may require the existence of integral membrane receptors that bind steroids at the cell surface and generate intracellular second messengers. This can be seen in highly specialized cells, such as spermatozoa, that are transcriptionally and translationally silent (Correia et al., 2007). This model has been called non-genomic signaling because it does not require transcription or translation to elicit its effect (Boonyaratanakornkit

and Edwards, 2007). The mPRs are also thought to signal via non-genomic mechanism because of their abilty to generate the second messenger sphingoid bases.

mPRα, mPRβ, and mPRγ are derived from genes located on chromosomes 1p36, 6p12, and 15q23, respectively that encode proteins of 346, 359, and 330 amino acids. Even though all three isoforms bind to progesterone, they are expressed in different tissues. The α-isoform is expressed in reproductive tissues, particularly in the placenta, testes, and ovaries, but also in the kidneys. The β-isoform is found only in the brain and the spinal cord, while the γ-isoform is present mainly in the kidneys and colon, but also found in the adrenal gland and lungs (Zhu et al., 2003a).

Human PAQR10 and PAQR11 proteins make up the final class of receptors. Their function is thought to be involved in monocyte to macrophage differentiation (Brauer et al., 2004). These proteins are distantly related to class I and II PAQRs; however, they are related to proteins from the bacterium, *Baciluus cereus,* named hemolysin III (Hly-III) (Baida and Kuzmin, 1996). Hly-III are known to have hemolytic activity and form pores in the outer membrane of the host cell (Baida and Kuzmin, 1996), but it is not known if this action occurs by direct or indirect means. Thus, more research is needed in this area to identify the function of receptors in this class.

Signaling of *FET3*

In our study, we will show a signaling mechanism in which increased PAQR gene dosage repressed *FET3* (Figure 1-11). This task was performed using a bottom up approach, where we first identified stress responsive transcriptional factors that are responsible for activation (Msn2p and Msn4p) and repression (Nrg1p and Nrg2p) of *FET3*. We will then identifiy several upstream signaling proteins required for this physiological effect. In particular, we will show the

involvement of AMPK, PKA, and the sphingoid base-sensing Pkh1p/Pkh2p. Finally, we will demonstrate that PAQR overexpression increases sphingoid bases, which, ultimately, leads to repression of *FET3*.

NRG and MSN

To survive in harsh conditions, many organisms have adapted to changes, including nutrient deficiency, changes in osmolarity, pH, temperature, and oxidative stress. *Saccharomyces cerevisiae* adapt by expressing different sets of genes (Mager and De Kruijff, 1995). One set of transcription factors that responds to stresses are the partially redundant (C_2H_2) zinc finger transcription factors *MSN2* (**M**ulti- **S**uppressor of *S**N**F1* mutation) and *MSN4* (Martinez-Pastor, et al., 1996). During times of stress such as heat shock, osmotic stress, and nutrient depletion, these transcription factors localize to the nucleus and activate stress responsive genes by binding to the stress response elements (STRE; CCCCT) in their promoters (Martinez-Pastor et al., 1996). Another set of transcription factors that regulate stress response genes are Nrg1p (**N**egative **R**egulator of **G**lucose-repressed genes) and Nrg2p. However, these proteins function as transcriptional repressors (Park et al., 1999). Nrg1p and Nrg2p bind to the NRE (**N**RG **R**esponse **E**lement; CCCTC) and are known to repress *FLO11* and *GAL* genes, which are implicated in glucose metabolism (Kuchin, et al., 2002) (Park et al, 1999). These transcription factors also negatively regulate haploid invasive growth as well as diploid pseudohyphal growth (Kuchin et al., 2002).

Protein kinase A (PKA) is known to be upstream of the stress response activators Msn2p and Msn4p, where PKA's signal causes the stress response to be down-regulated (Boy-Marcotte et al., 1998). This occurs by PKA phosphorylating Msn2p, which causes it to accumulate in cytoplasm and stop the stress response (Smith et al, 1998).

Upstream of the Nrg1p and Nrg2p proteins is Snf1p, the catalytic subunit of the AMP-dependent kinase complex. The binding sites for Nrg1p/Nrg2p and Msn2p/Msn4p are very similar. Recent studies suggest these two sets of transcription factors compete for binding sites on a subset of stress response promoters (Vyas et al., 2005). The CCCTC site can also be found in the promoter regions of both *FET3* and *ZRT1*, which may implicate these transcription factors in the regulation of iron and zinc homeostasis.

AMPK

The **AMP**-**k**inase (AMPK) complex is a heterotrimer composed of a catalytic α-subunit, β-subunit, and a regulatory γ-subunit (Figure 1-12). In *Saccharomyces cerevisiae* the *SNF1* (**S**ucrose **N**on-**F**ermenting) gene encodes, the catalytic α-subunit and is a serine/threonine protein kinase that is activated during times of glucose limitation (Woods et al, 1994). It is required for expression of glucose repressed genes involved in utilization of galactose, sucrose, raffinose, or nonfermentable carbon sources, as well as respiration, gluconeogenesis, filamentation and sporulation (Celenza and Carlson, 1986) (Thompson-Jaeger et al, 1991). Snf1p has been found to interact with other proteins including the γ-subunit Snf4p. Snf4p is also required for transcription of glucose repressed genes and functions as a Snf1p activator (Celenza and Carlson, 1989). The β-subunits interact with both Snf1p and Snf4p, and act as a scaffold to hold the heterotrimer complex together. *S. cerevisiae* express three β-subunits encoded by the *GAL83* (**GAL**actose metabolism), *SIP1* (***S****NF1* **I**nteracting **P**rotein), and *SIP2* genes. At least one of the β-subunits needs to be expressed with each subunit conferring functional specificity to Snf1p (Schmidt and McCartney, 2000). However, the major role of the β-subunits is to control localization of the AMPK complex (Vincent et al, 2001). During growth in high glucose media, all three of the β-subunits are localized to the cytoplasm, but when yeast are switched to non-fermentable carbon sources, Gal83p-Snf1p moves to the nucleus, Sip1p-Snf1p shifts to the

vacuole membrane, and Sip2p-Snf1p remains in the cytoplasm. Activation of AMPK occurs by three upstream kinases, known as Sak1p (**S**nf1p **A**ctivating **K**inase), Tos3p (**T**arget **O**f **S**BF), and Elm1p (**EL**ongated **M**orphology).

PKA

Protein kinase A (PKA, cAMP-dependent kinase) plays a major role in metabolism and cell proliferation based on nutrient availability. For PKA activation, a high level of cAMP is required and occurs by Ras2p through the activation of adenylate cyclase. PKA is activated by cAMP binding to the regulatory subunit Bcy1p (**B**ypass of **CY**clase mutations), which allows the release of three catalytic subunits encoded by *TPK1* (**T**akashi's **P**rotein **K**inase), *TPK2*, and *TPK3* genes (Thevelein, 1994). Furthermore, PKA regulates genes involved in iron uptake and respiration; however, each catalytic subunit has its own function. Tpk2p represses genes involved in high-affinity iron uptake but induces pseudohyphal growth. Tpk1p derepresses genes involved in branched chain amino acid formation and also has a role in controlling in iron levels (Robertson et al., 2000).

PDK (*PKH1* and *PKH2*)

The next step was to find what enzymes act upstream of PKA and AMPK. A clue came from trying to elucidate the *IZH* genes, where *IZH1* and *IZH4* were found to be divergently transcribed from a pair of kinases, *PKH1* and *PKH2* (Figure 1-13). In yeast, genes that share the same promoter are often co-regulated and belong to the same chemical pathway (Kruglyak and Tang, 2000)

Pkh1p (**P**kb-activating **K**inase **H**omolog) and Pkh2p are partially redundant isoforms that are homologs of human PDK1 (**P**hosphoinositide **D**ependent **K**inase) (Casamayor et al, 1999). PDK1 is a member of the AGC family of kinases, that is a main regulator of downstream

kinases, such as PKB (**P**rotein **K**inase **B**), S6K (p70 ribosomal **S6** **K**inase), and SGK (**S**erum- and **G**lucocorticoidstimulated protein **K**inase) (Vanhaesebroeck and Alessi, 2000).

But how are Pkh1p and Pkh2p activated? In humans, PDK is activated by four signals: autophosphorylation, 3-phosphoinositides, *TOR2*, and sphingosine (Kim et al., 2002). But in yeast, the lipid (phyto)sphingosine is the only known activator of both Pkh1p and Pkh2p (Liu et al., 2000).

Sphingolipids

Sphingolipids are one of the main lipids comprising membranes in eukaryotic cells. But sphingolipids also play a role as second messengers in regulating signal transduction pathways, mediating cell growth, differentiation, and in cell death (Futerman and Riezman, 2005). Importantly, the ratio of sphingosine to ceramide inside the cell may act as a rheostat where sphingosine controls cell proliferation and ceramide has roles in apoptosis (Pyne and Pyne, 2000). To understand specific functions of sphingolipids and their derivatives, especially the identification of genes involved in sphingolipid metabolism, many researchers are using the simple model organism, *Saccharomyces cerevisiae* (Dickson and Lester, 2002) (Sims et al, 2004).

Structurally, sphingolipids are composed of a long chain base (LCB), a fatty acid, and a polar head group. In yeast, two kinds of LCBs can be found, dihydrosphingosine (DHS) and its 4-hydroxy derivative, phytosphingosine (PHS).

The first and rate limiting step in sphingolipid synthesis (Figure 1-14) is the formation of 3-ketodihydrosphingosine from palmitoyl-CoA and serine. This reaction, which occurs on the cytoplasmic surface of the endoplasmic reticulum (ER) (Mandon et al., 1992), is catalyzed by the two subunit (Lcb1p (**L**ong **C**hain **B**ase) and Lcb2p) serine palmitoyltransferase (Buede et al., 1991; Pinto et al., 1992) Yeast cells defective in *LCB1* and *LCB2* genes are lethal and cannot

grow without LCB supplementation (Wells et al., 1998). But in humans a mutation in *LCB1* causes sensory neuropathy, a disease that causes degeneration of the dorsal root ganglia and motor neurons (Gable et al., 2002). Tsc10p catalyzes the second step in which 3-ketodihydrosphingosine is reduced to DHS (Beeler et al., 1998). Then, two ceramide synthases, Lag1p (**L**ongevity **A**ssurance **G**ene) and Lac1p (**L**ongevity **A**ssurance gene **C**ognate) (Guillas et al., 2001), react with DHS and a C26 fatty acid CoA to make dihydroceramide. Dihyroceramide can then be hydroxylated at C4 by Sur2p (**SU**ppressor of **R**vs161 and rvs167 mutations) to yield phytoceramide. However, phytoceramide can also be formed from DHS by Sur2p to form PHS, then amide linked to the C26 fatty acid. Further modification can occur when yeast are grown in the presence of oxygen, where the enzyme Scs7p hydroxylate the fatty acid group at C2 and C3 positions (Haak et al., 1997).

An important aspect of the ceramide synthesis is the formation of C26 fatty acids from C14-C18 fatty acids. These reactions take place in the ER and require several genes, including *ELO1* (**ELO**ngation defective (Toke 1996), *FEN1* (**FEN**propimorph resistance), *SUR4* (Oh 1997), *TSC13* (**T**emperature-**S**ensitive **C**sg2 suppressor) (Kohlwein et al., 2001), *YBR159w* (Han 2002 JBC), and *ACP1* (**A**cyl **C**arrier **P**rotein) (Gaigg et al., 2001). Once C14 and C16 fatty acid are formed from acyl-CoA, Elo1p starts enlongating the fatty acid chain. Fen1p then performs the condensation reaction with C20 and C22 fatty acyl-CoAs, while Sur4p does the same for C22 and C24 fatty acyl-CoAs (Rossler et al., 2003). Ybr159w is proposed to reduce 3-ketoacyl-CoA to 3-hydroxyacyl-CoA for fatty acids greater than 18 carbons (Han et al., 2002); however, the protein that is involved in dehydrating 3-hydroxyacyl-CoA to the enol intermediate is unknown. The next step is the elongation of the fatty acid catalyzed by Tsc13p, which reduces the enol.

Finally, Acp1p is needed in this process to help deliver the fatty acid CoA to start the process of elongation.

Once ceramides are synthesized, they are transported from the ER to the Golgi apparatus where the polar head substituents can be added at C1. In human cells there are hundreds of sphingolipid molecules that differ in their head group. But in yeast there are only three classes of sphingolipids. The simplest yeast sphingolipid, inositolphosphorylceramide (IPC), is made by transferring inositolphosphate from phosphatidate to ceramide by the enzyme Aur1p (**AU**reobasidin A **R**esistance) (Hashida-Okado et al., 1996). Subsequent maturation of IPC requires Sur1, Csg2 (**C**a^{2+} **S**ensitive **G**rowth), and Csh1 (***C****SG1*/***S****UR1* **H**omolog), which adds mannose to form mannosyl-inositolphosphorylceramide (Zhao et al., 1994). Finally, MIPC is converted to mannosyl-diinositolphosphorylceramide ($M(IP)_2C$) by Ipt1p (Dickson et al., 1997). Once formed, the three classes of sphingolipids can localize to plasma membrane where they make up 30% of all the lipids (Patton and Lester, 1991).

The breakdown of sphingolipids, especially in mammals, is essential for survival. Many diseases occur due to lack of turnover of one or more sphingolipids (Raas-Rothschild et al., 2004). Breakdown begins with Isc1p (**I**nositol phospho**S**phingolipid phospholipase **C**) hydrolyzing IPC, MIPC, and $M(IP)_2C$'s polar head group to yield ceramide (Ella et al., 1997) (Sawai et al., 2000). Isc1p in S. cerevisiae plays a role in stress tolerance as sodium chloride and lithium chloride have been shown to cause growth defects in an *isc1*-deleted strain (Betz et al., 2002).

Ceramides, whose concentration levels are controlled by *de novo* synthesis, have many roles ranging from apoptosis to stress responses (Ogretmen and Hannun, 2004). However the inability to deacylate the amide bond between sphingosine and fatty acid in humans leads to

lysosomal storage condition known as Farber's disease (Koch et al., 1996). In yeast, Ypc1p (**Y**east **P**hyto**C**eramidase) and Ydc1p (**Y**east **D**ihydro**C**eramidase) are the ceramidases that yield either PHS or DHS (Mao et al., 2000).

PHS or DHS derived from either *de novo* synthesis or ceramide breakdown can be phosphorylated by two LCB kinases, Lcb4p and Lcb5p (Nagiec et al., 1998), to give PHS-1-phosphate (PHSP) and DHS-1-phosphate (DHSP). Two fates arise for PHSP and DHSP. One, they can be catabolized by Dpl1p (Saba, et al., 1997) to yield ethalomine-phosphate and fatty aldehydes. Two, they can be dephosphorylated by Lcb3p and Ysr3p (**Y**east **S**phingolipid **R**esistance) (Qie et al., 1997; Mao et al., 1997) to again form PHS and DHS. Functionally, PHSP and DHSP are recognized as signaling molecules and mediate processes such as cell growth and survival and calcium mobilization (Pyne and Pyne, 2000) (Spiegel and Milstien 2003).

Yeast as a Model Organism

Saccharomyces cerevisiae, commonly known as baker's, brewer's, or budding yeast, has been a standard laboratory model since the 1950s. Yeast has many attributes in biological studies such as being a hearty organism, exhibiting rapid growth, having the ease of replica plating and mutant isolation, a well-defined genetic system, and being a versatile DNA transformation system (Gietz and Woods, 2002). Additionally, yeast is a non-pathogenic organism and can be handled with little precaution.

In contrast to other organisms, *Saccharomyces cerevisiae* can exist in either a haploid or diploid state, and are viable with a large number of markers. The development of DNA transformation has made yeast an ideal organism for gene cloning and genetic engineering techniques. Structural genes corresponding to almost any genetic trait can be identified from complementation from plasmid libraries. Genes can be expressed in yeast cells through

replicating plasmids or by integration of the gene into the genome. Unlike many other organisms, recombination of transforming DNA in yeast occurs by homologous recombination. Thus, cloned yeast sequences, along with foreign sequences on plasmids, can be directed to specific location in the genome. This allows the user to replace a certain gene with its mutated counterpart, which enables the researcher to make a mutant library. In fact, researchers can use an abundance of tools that are commercially available, such as a full set of deletion mutants and tagged open reading frames to localize or purify the protein of choice.

In using yeast, several genetic databases exist, which is an important tool for developing basic knowledge about the function and organization of eukaryotic cell genetics and physiology. Also, a large group of yeast researchers contribute a wealth of information and expertise.

Saccharomyces cerevisiae was the first eukaryote whose genome was completely sequenced (Goffeau, et al., 2006). Thus, yeast has become one of the most important organisms for genomic research (Kumar and Snyder, 2001), and has been useful for studying protein-protein interaction by two hybrid analysis (Uetz et al., 2000), protein localization (Ross-MacDonald et al., 1999), enzymatic activities (Martzen et al., 1999), genome-wide analysis of gene function by gene disruption (Oliver, 1996), and serial analysis of gene expression (Velculescu et al., 1997).

One advantage of using yeast is that mammalian genes can be introduced into yeast for functional analyses of the corresponding gene products. At this time, there have been 6,000 genes recognized in yeast while, over 25,000 have been discovered in humans (Goffeau et al., 2006). Intriguingly, one-third of yeast proteins share homology to human proteins and 34% of disease-related human genes have functional homologs in yeast (Sturgeon et al., 1996). This

high conservation of metabolic and regulatory mechanisms has contributed to yeast becoming the model eukaryotic system for diveresed biological studies.

Additionally, yeast has been used to elucidate a variety of basic biological processes due to their simplicity of experimentation. For metal ion homeostasis, organisms have evolved complex mechanisms that have been conserved throughout evolution. Multiple transport systems exist for these metals allowing organisms to obtain these metals under varying conditions. Yeast is an ideal model system because many of the individual proteins and pathways are conserved from yeast to humans (Valentine and Gralla, 1997). Thus, the information gained can have clinical applications such as improving dietary zinc and iron availability in humans and treating metal-related disorders (De Freitas et al., 2003).

Summation

In our initial studies, we examined several yeast proteins that were implicated in zinc homeostasis; however, as we studied these proteins more closely we saw they also acted in iron homeostasis, as well as lipid metabolism and mediating signaling events. We also noticed that the yeast proteins were related to eleven human proteins that bind to ligands such as adiponectin and progestin. We hypothesize that overexpression of the protein or ligand binding to its receptors will hydrolyze phytoceramide to phytoceramide, which in turn will activate Pkh1p/Pkh2p, protein kinase A and deactivate AMPK that will result in repression of the high affinity iron and zinc uptake systems by activating the repressors, Nrg1p/Nrg2p, and deactivating the activators, Msn2p/Msn4p (Figure 1-11). The work outlined in this study will focus on the proteins involved in the high affinity iron and zinc uptake system, as well as the proteins participating in this signaling pathway. The focus of this work entails studying ligand binding to its receptors. We also studied exogenous and endogenous sphingoid lipids and their effects on these uptake systems. By investigating these membrane protein receptors in yeast, we may be

able to understand their effect on metal homeostasis and may one day apply this information to mammalian systems.

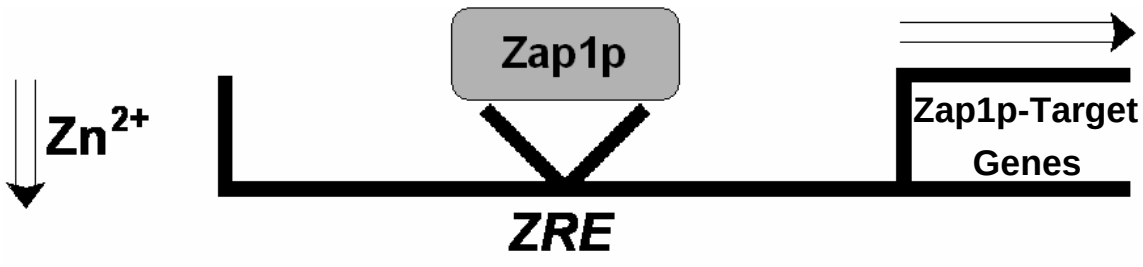


Figure 1-1. The transcription factor Zap1p binds to the zinc response element (ZRE) on the promoter region of genes in *Saccharomyces cerevisiae* during periods of low zinc.

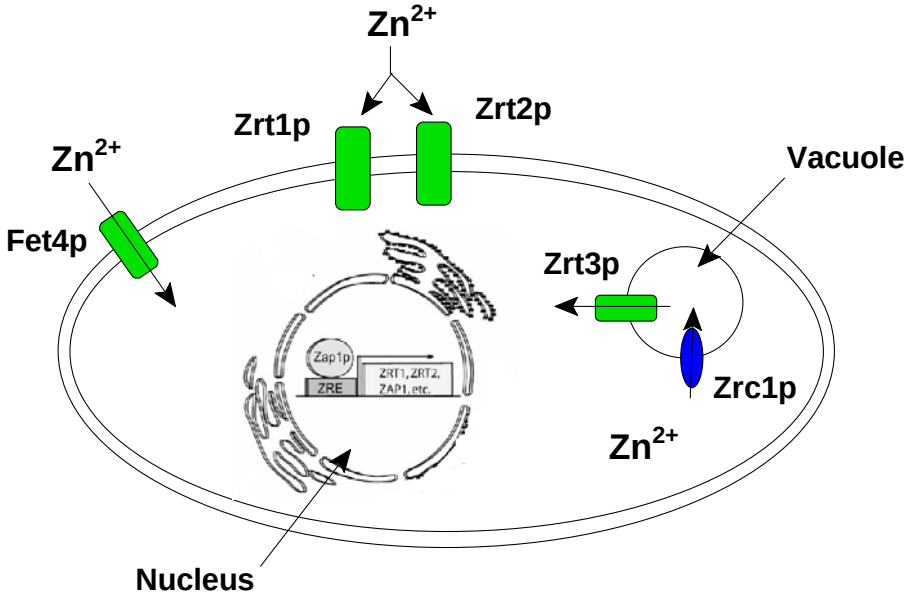


Figure 1-2. A *Saccharomyces cerevisiae* cell showing the zinc transporters.

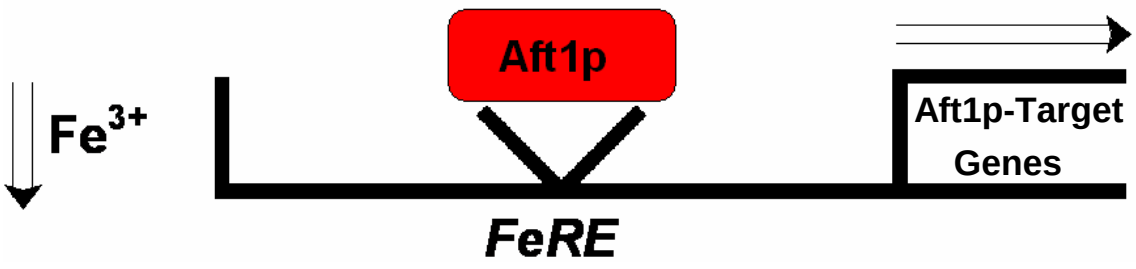


Figure 1-3. The transcription factor Aft1p binds to the iron response element (FeRE) on the promoter region of genes in *Saccharomyces cerevisiae* during periods of low iron.

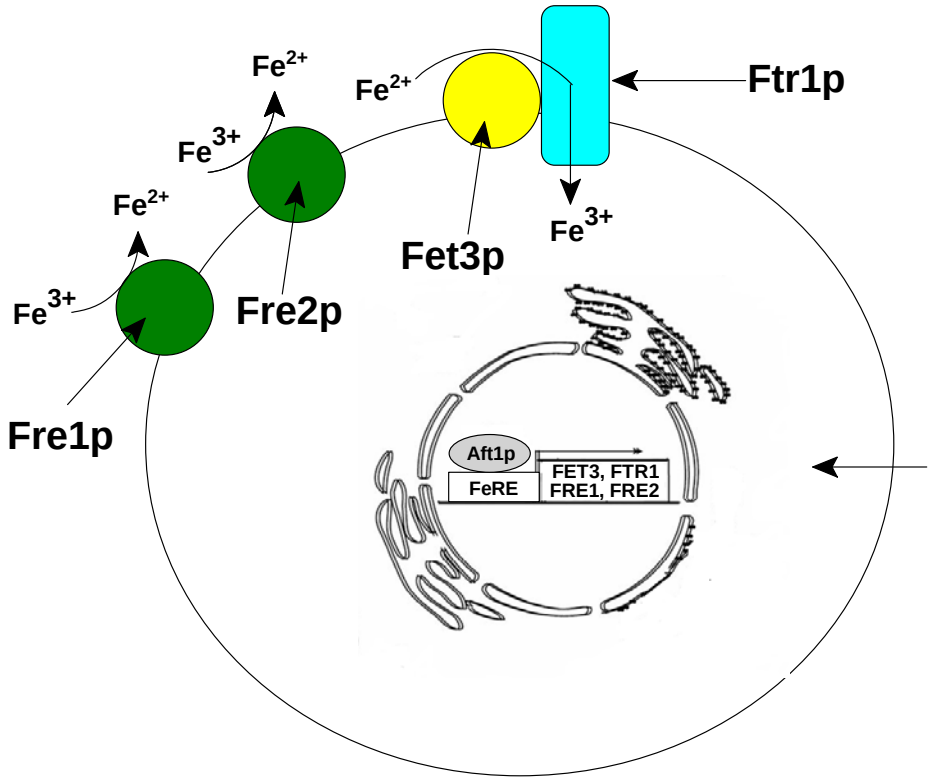


Figure 1-4. A *Saccharomyces cerevisiae* cell showing the iron transporters.

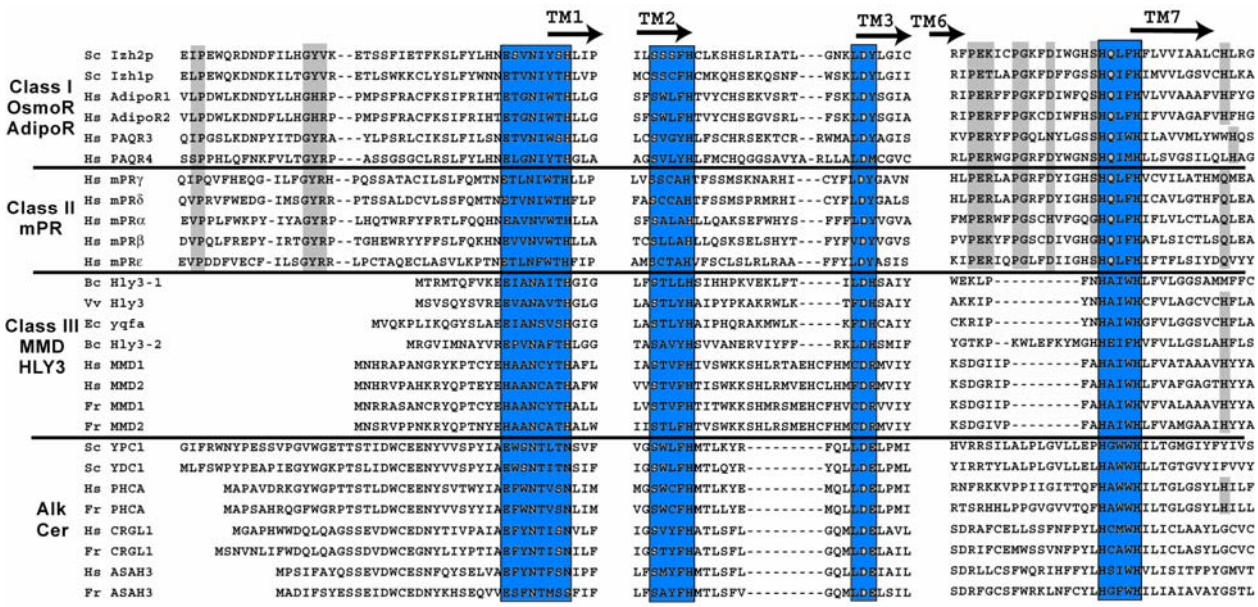


Figure 1-5. Multiple sequence alignment of PAQR proteins. Areas highlighted are highly conserved motifs.

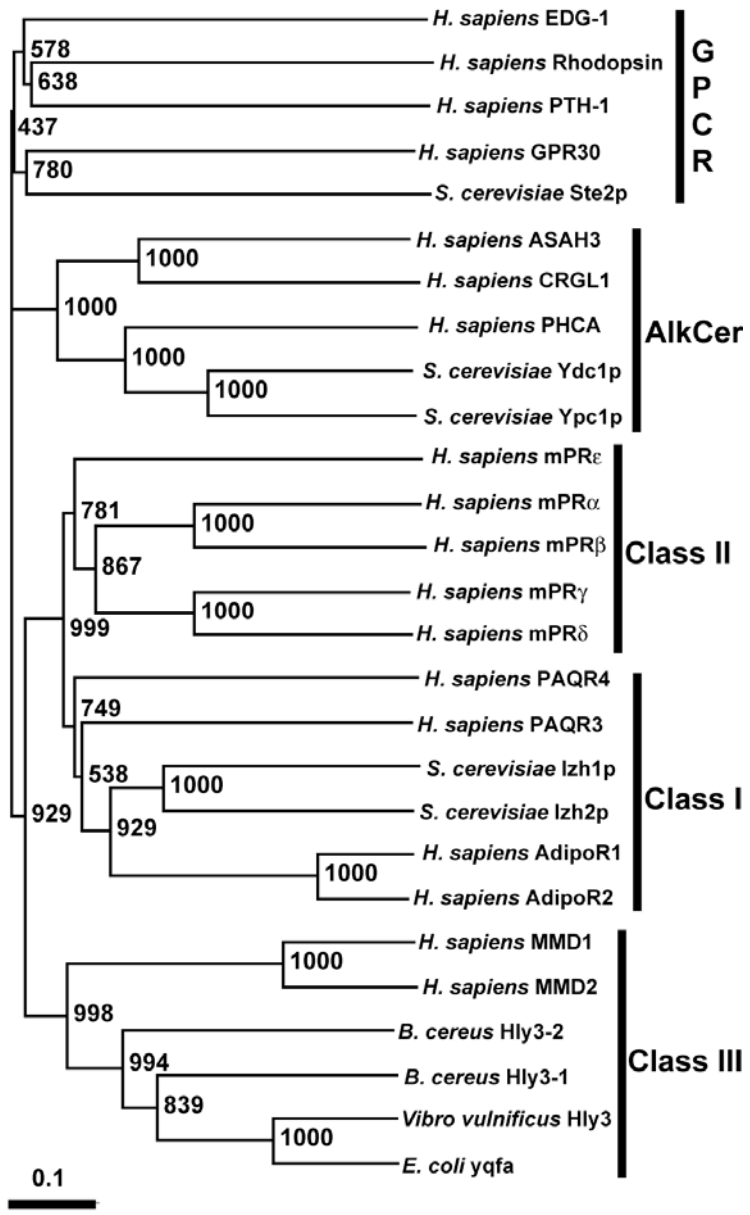


Figure 1-6. Phylogenetic tree displaying *IZH* and human PAQR proteins. A bootstrapped phylogentic tree showing the relationship between PAQRs, alkaline ceramidases and GPCRs. Yeast Izh proteins cluster with human adiponectin receptors. The GPCRs clade is included as an outgroup to root the phylogentic tree. The length of the tree branches is proportional to the calculated distance between sequences with the scale bar indicating 0.1 substitutions per site. Number at the nodes are confidence values that refer to the number of times per 1,000 trees drawn a particular grouping is made.

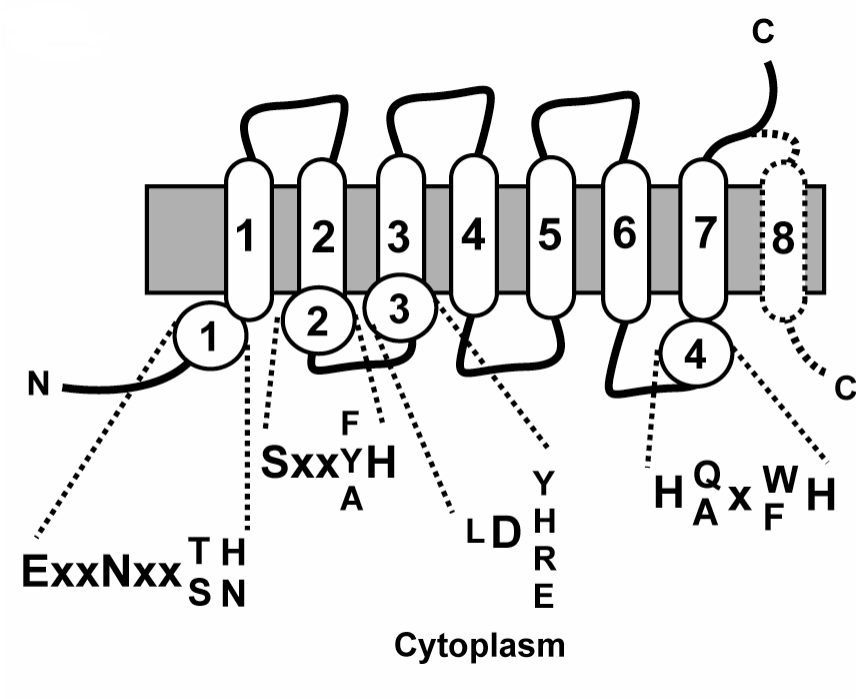


Figure 1-7. Topology predicted for all members of the PAQRs with the locations and the consensus sequences of the three highly conserved motifs. Grey represents membrane, ovals indicate transmebrane domains, and circles represent conserved regions of PAQR family. Class I and III PAQRs are presumed to have 7 transmembrane domains while Class II PAQRs may have 8 transmembrane domains.

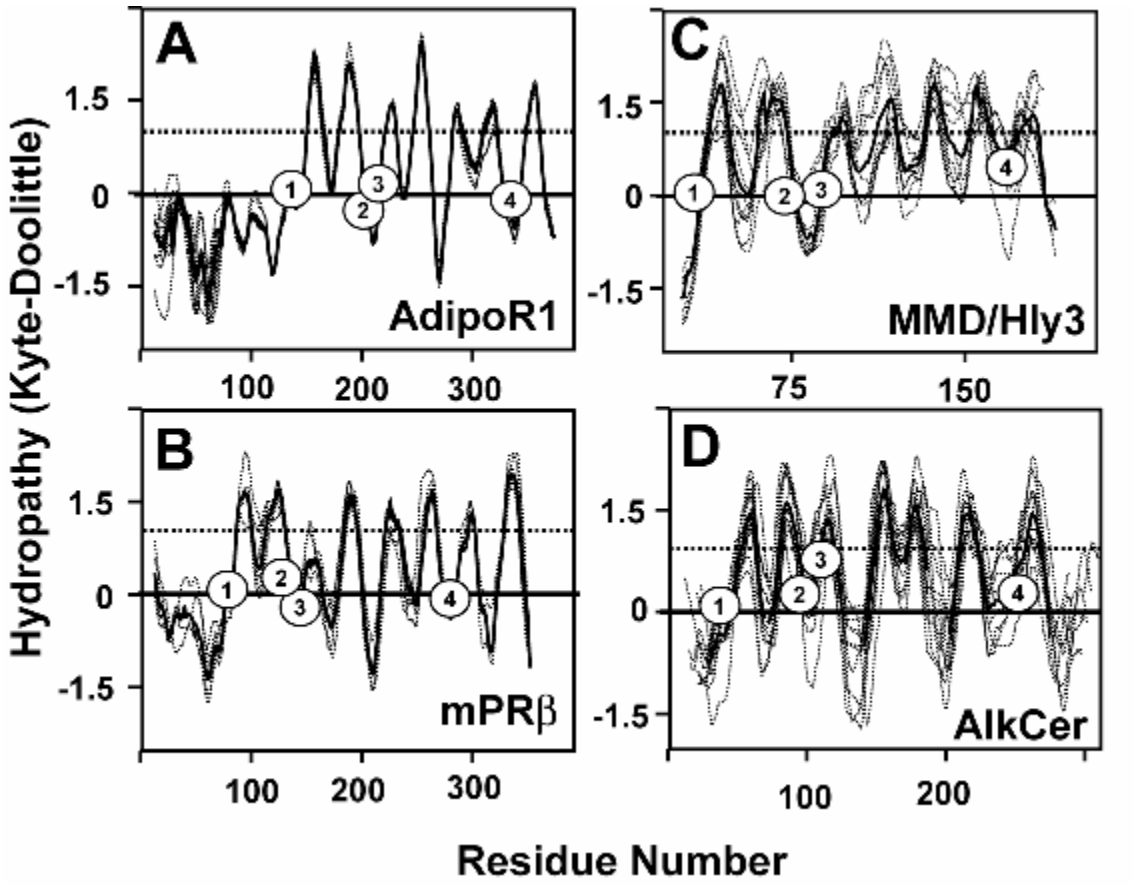


Figure 1-8. Kyte-doolittle plot of PAQR and PAQR-like proteins. (A) Class I PAQR – vertebrate homologs of AdipoR1. (B) Class II PAQR – vertebrate homologs of mPRβ. (C) Class III PAQR – homologs of PAQR11. (D) Alkaline ceramidases.

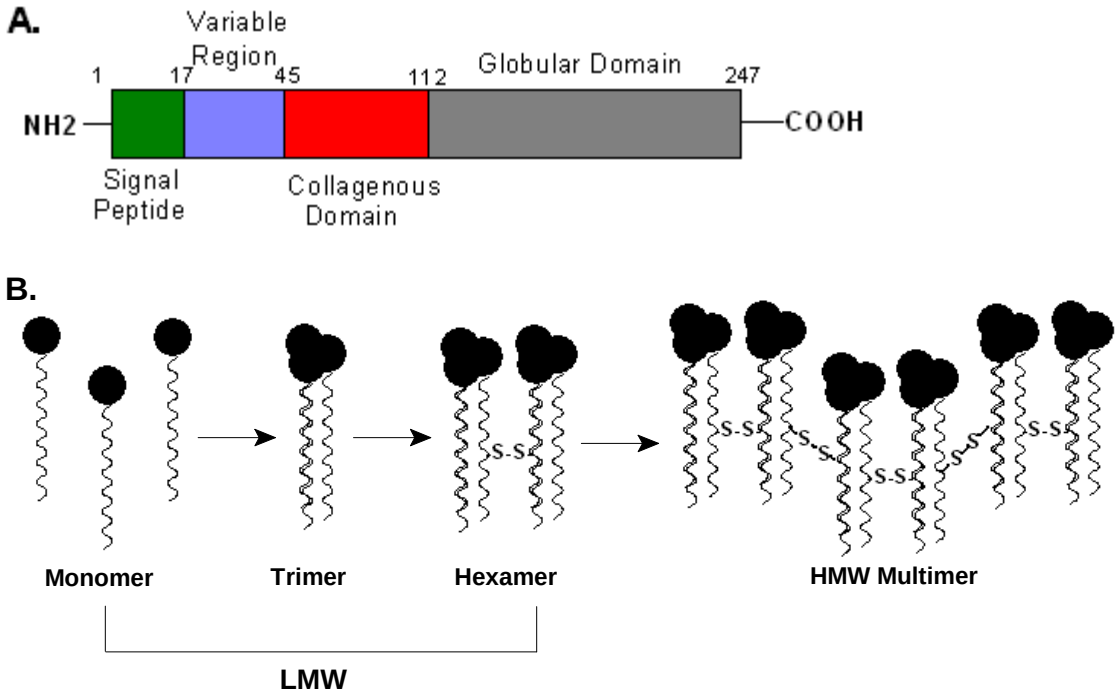


Figure 1-9. Adiponectin domains and forms. (A) Human adiponectin domain structure. Numbers indicate amino acids in each region. (B) Multimer formation of adiponectin.

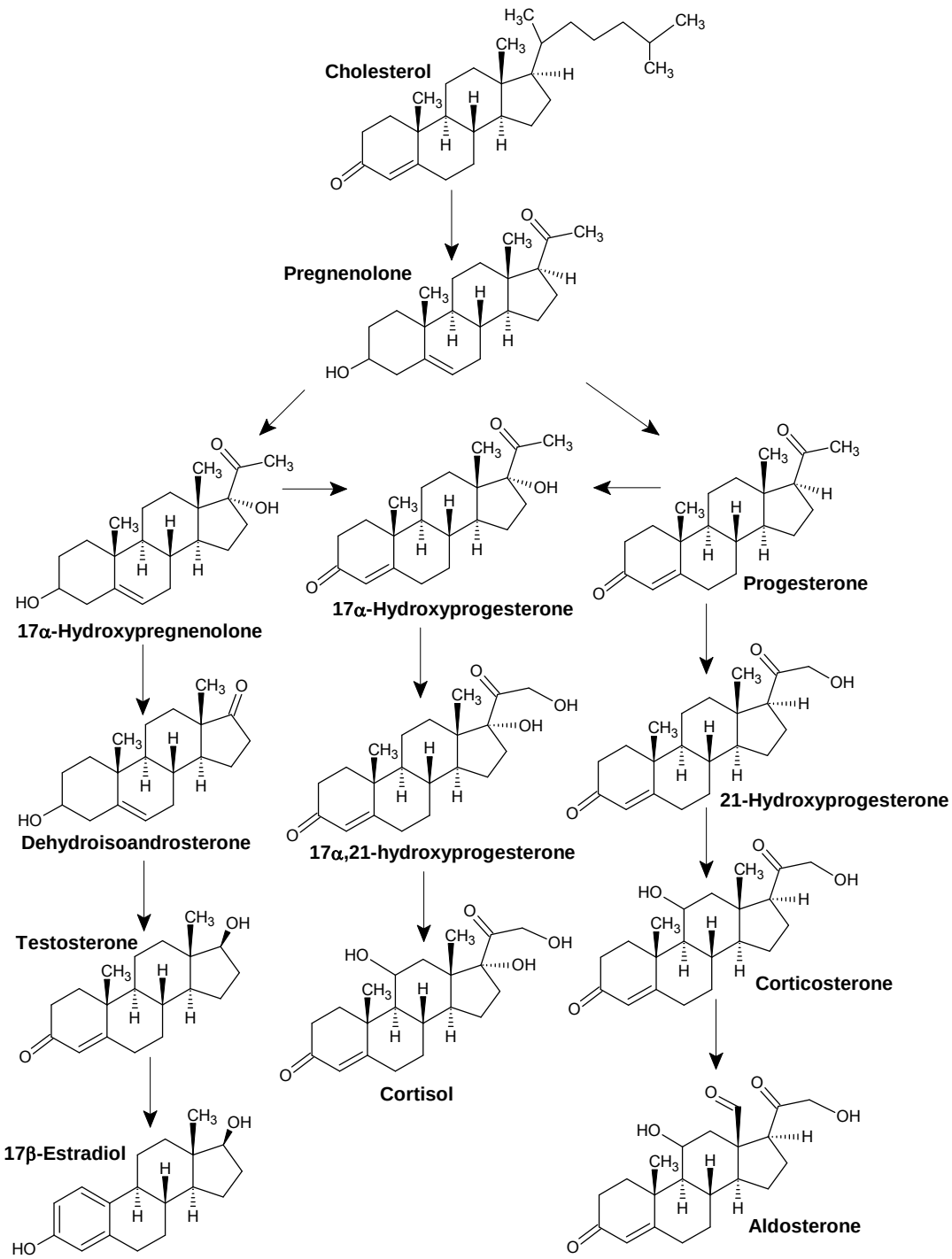


Figure 1-10. Synthesis of progestins, glucocorticoids, mineralocorticoids, androgens, and estrogens. All steroids synthesized are from pregnenolone via cholesterol.

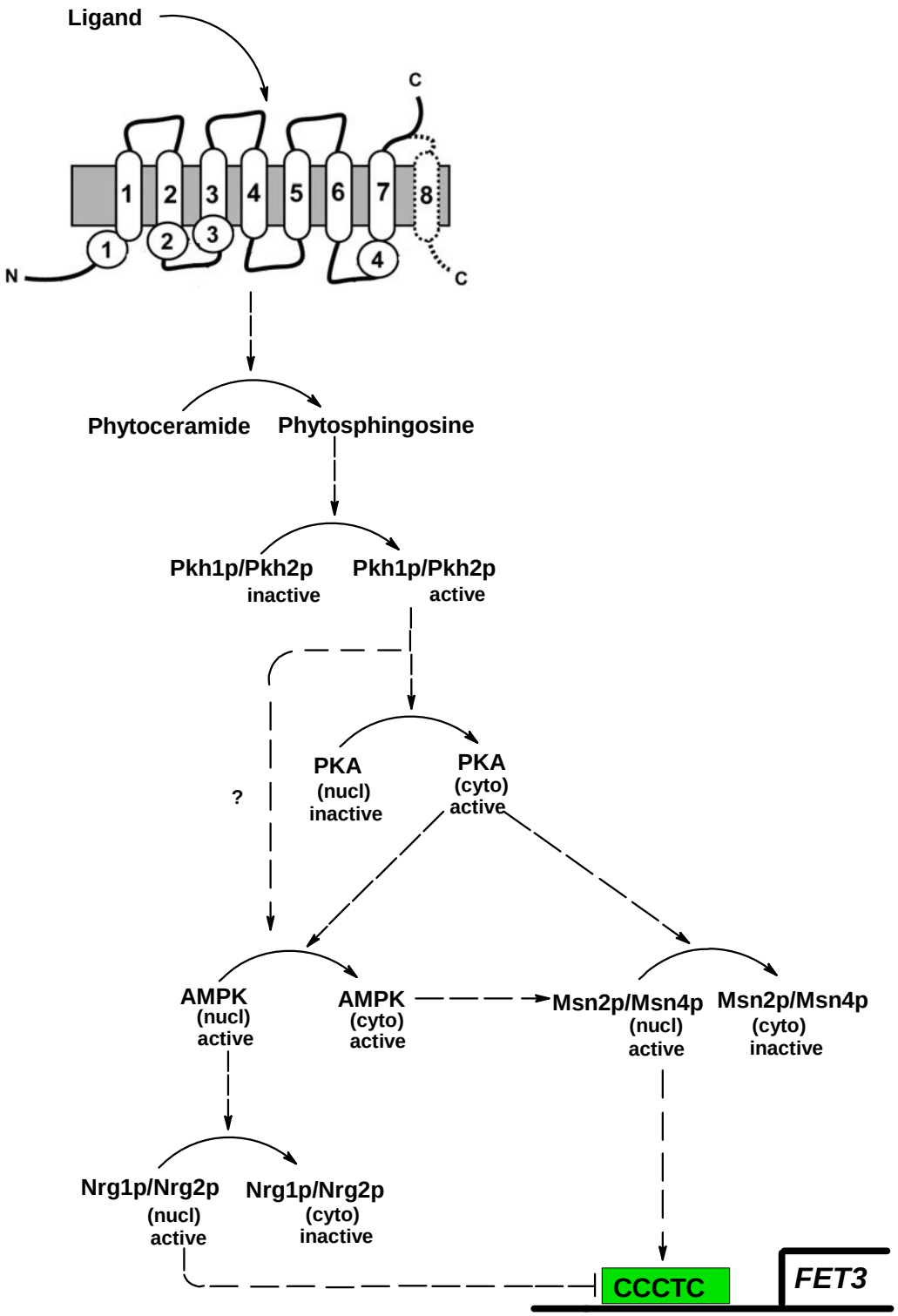


Figure 1-11. A model for the mechanism of *FET3* by PAQR overexpression. Solid arrows indicate protein activation or localization of protein. Broken arrows indicate protein interaction on localization and activation. Cyto = protein is cytoplamic. Nucl = protein is nuclear.

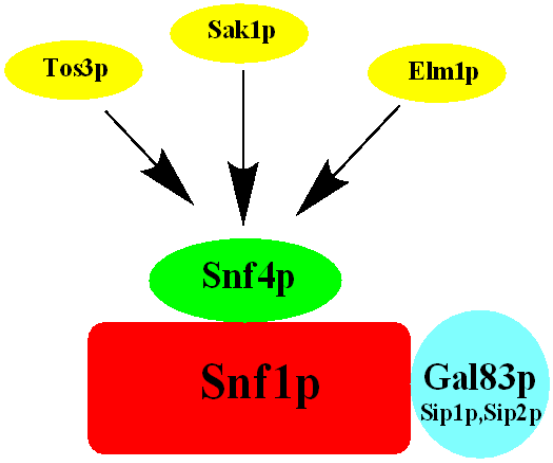


Figure 1-12. The AMPK complex and activators. The AMPK complex is heteorotrimer composed of an α-subunit (Snf1p), γ-subunit (Snf4p), and one of three β-subunits (Gal83p, Sip1p, and Sip2p). Snf1p is activated by one of three AMPK kinases (Elm1p, Tos3p, and Sak1p).

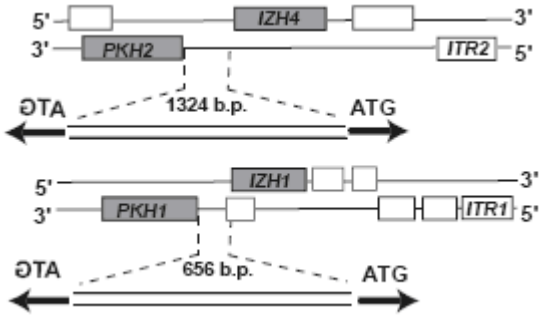


Figure 1-13. Saccharomyces cerevisiae genes *IZH1* and *IZH4* are divergently transcribed from *PKH1* and *PKH2*.

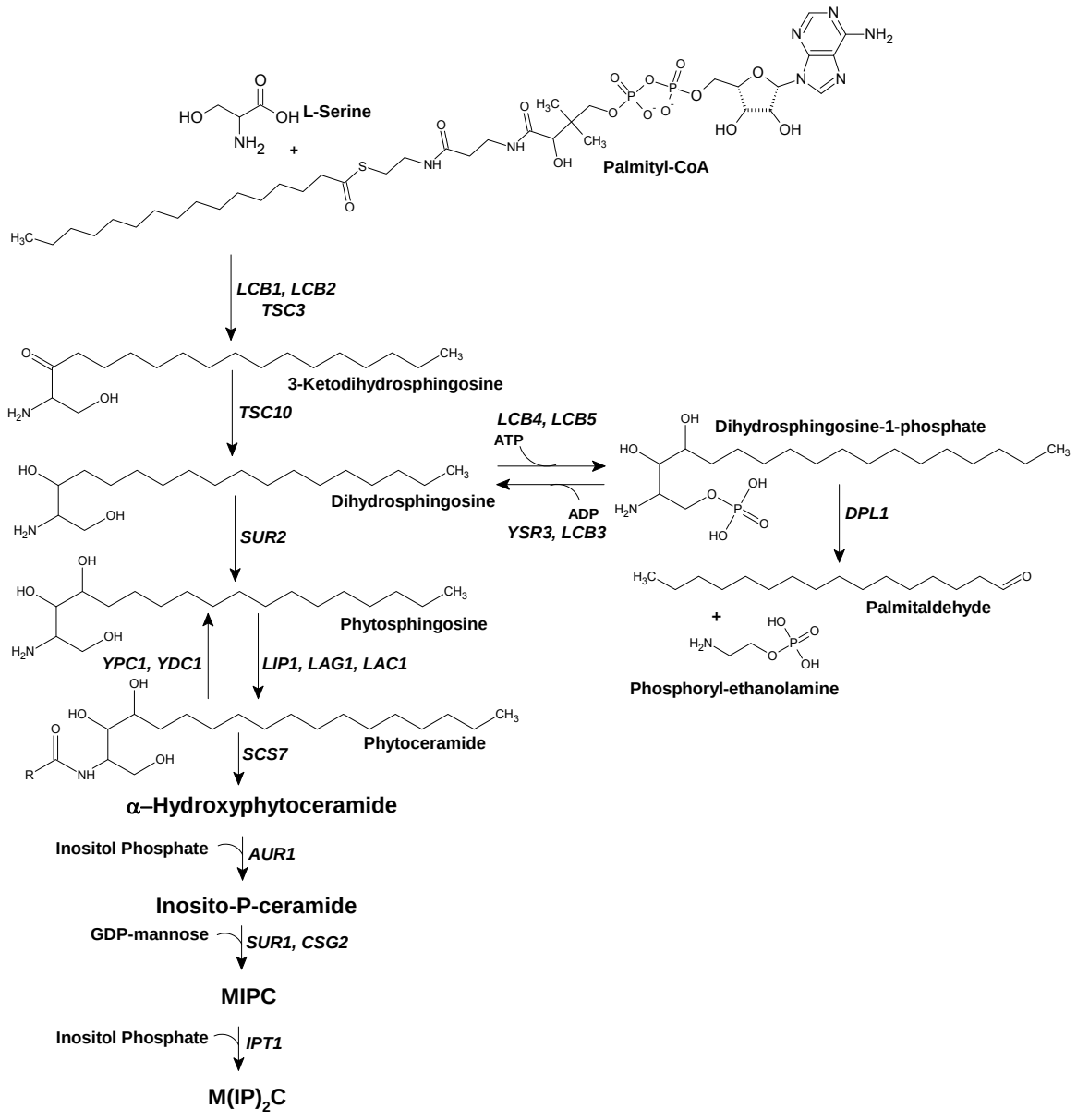


Figure 1-14. Sphingolipid biosynthesis in *Saccharomyces cerevisiae*. Not shown, phytosphingosine like dihydrosphingosine can be degraded in a similar manner to palmitaldehyde and phosphoryl-ethanolamine.

CHAPTER 2
THE EFFECT OF *IZH2* ON *FET3* AND *ZRT1*

Introduction

Iron and zinc are essential nutrients and play important roles in many biochemical processes. Zinc is a catalytic component of over 300 enzymes and has structural roles in many proteins, while iron can donate and accept electrons and is a cofactor in many redox-active metalloenzymes (Eide, 2001). However, when these metals become scarce, an organism must find ways to increase uptake of iron and zinc to maintain growth. Therefore, organisms have evolved homeostatic regulatory systems to control uptake during nutrient depletion (Rutherford and Bird, 2004).

In *Saccharomyces cerevisiae*, iron- and zinc-acquisition during periods of limitation are controlled by the transcription factors, Aft1p and Zap1p. Aft1p and Zap1p sense iron and zinc in response to deficiency, which induce genes involved in uptake. However, recent studies have showed iron and zinc uptake systems may be induced during iron and zinc sufficiency when Aft1p and Zap1p are turned off. The high affinity iron- and zinc-uptake systems are also regulated by carbon starvation (Haurie et al., 2003) and during different periods of the cell cycle (Cho et al., 1998).

During severe iron limitation, *Saccharomyces cerevisiae* induces genes involved in iron-acquisition by Aft1p. Iron is taken into the cell by the high affinity transport system (Stearman et al., 1996). Since iron in the environment is found as insoluble Fe^{3+}, it must be reduced to Fe^{2+}, which occurs by two flavocytochromes containing ferrireductases, Fre1p and Fre2p (Georgatsou and Alexandraki, 1994). Fe^{2+} is then handed over to Fet3p, a ferroxidase, which reoxidizes the iron. Finally, Ftr1p transports Fe^{3+} across the cell membrane and into the cell (Askwith et al., 1994). Zinc uptake in *S. cerevisiae* is controlled by Zap1p in response to zinc levels inside the

cell. During zinc limitation, *ZRT1*, the high affinity zinc transporter is induced (Zhao and Eide, 1996), while the low affinity zinc transporter is induced by low zinc levels and controlled by *ZRT2* transcription.

Recent studies have identified four proteins that have been implicated in metal ion homeostasis and named *IZH1-4* (**I**mplicated in **Z**inc **H**omeostasis) (Lyons et al., 2004). *IZH2* functions as a receptor for the plant defensin, osmotin, but it is still not known if this represents its physiological role (Narasimham et al., 2001). Also, *IZH1* and *IZH2* are confirmed targets of Zap1p, a transcription factor that induces genes during zinc limitation. Additionally, *IZH2* and *IZH4* are positively regulated by excess metals via Mga2p (Lyons et al., 2004). The Izh proteins belong to a larger family of membrane receptor proteins known as the **P**rogestin and **A**dipo**Q** **R**eceptors (PAQRs).

In this study, we present data showing that overexpression of the *IZH2* gene resulted in repression of a Zap1p-responsive reporter. For this reporter, a **Z**inc **R**esponsive **E**lement (ZRE) (Zap1p binding site located in the promoter) from the ZRT1 promoter was inserted into a minimal *CYC1* reporter. This reporter responds to zinc deficiency, but overexpression of *IZH2* repressed this reporter. Control experiments demonstrated that the effect of *IZH2* overexpression on this reporter was not due to a generalized defect in transcription, translation, or aberrant trafficking of membrane proteins due to their overexpression. Additionally, we did not see an increase in zinc levels inside the cell, which suggests this effect was due to a Zap1p independent mechanism (Lyons et al., 2004).

We also showed that *IZH*-dependent repression of the reporter may be due to a regulatory element, CCCTC, which overlaps the inserted ZRE. Furthermore, we demonstrate the full length *ZRT1* reporter is also affected by *IZH2*-dependent repression. Since *IZH2* overexpression affects

genes involved in high affinity zinc uptake, we decided to examine their effect on genes involved in high affinity iron uptake. We present data that *IZH2* overexpression represses *FET3* during iron limitation. Furthermore, the CCCTC motif, which is a known NRG binding site (Park et al., 1999), is also found in the *FET3* promoter (Kupchak et al., 2007). We suggest that repression of *FET3* occurs by activation of the repressors Nrg1p/Nrg2p and deactivation of the activators Msn2p/Msn4p (Figure 2-1), which may compete for binding to the CCCTC site.

This study suggests that *IZH2* overexpression affects high affinity iron and zinc transporters during iron- and zinc-limitation. Also, this repression may be due to a regulatory element, CCCTC, found inside the ZRE and *FET3* promoter.

Materials and Methods

Yeast Strains and Plasmids

The wild type strain (DY1457) was a gift of Dr. David Eide (University of Wisconsin, Madison). The BY4742 wild type strain and the *msn2Δ*, *msn4Δ, nrg1Δ,* and *nrg2Δ* mutant strains were purchased from Euroscarf (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/col_index.html), while the wild type strain MCY5326 and *msn2Δmsn4Δ, nrg1Δnrg2Δ,* and *nrg1Δnrg2Δmsn2Δmsn4Δ* mutant strains were provided by Dr. Marian Carlson at Columbia University (Vyas, et al., 2005) The genotype of all strains are listed in Supplementary Table 1. The p*FET3-lacZ* plasmid (Gift of D. Eide) has the promoter region of the *FET3* gene fused to the *lacZ* open reading frame and is *URA3* or *LEU2* selectable. The p*FLO11-lacZ* strain, in which the *lacZ* ORF has replaced the *FLO11* ORF in the genome 1278b, was provided by Dr. Florian Bauer at the University of Stellenbosch, Matieland, South Africa (van Dyk, 2005). The p*FET3-lacZ* plasmid has the promoter region of the p*FET3* gene fused to the *lacZ* open reading frame and is *URA3* selectable. p*CYC-STRE* (pCZ-oligo31/32, provided by Janet Treger, UCLA) contains a fragment of the DDR2 promoter that includes tandem stress response elements

(STRE) to which the Msn2p and Msn4p transcriptional factors bind (Treger et al., 1998). *IZH2* was cloned from yeast genomic DNA into pRS316-*GAL1* (*LEU2*) via homologous recombination as previously described (Lyons et al., 2004). The primers used to clone *IZH2* are listed in Supplementary Table 2. Plasmids containing the *GAL1*-driven TAP (Tandem Affinity Purification) tagged *NRG1* and *NRG2* constructs were purchased from Open Biosystems. All clones were sequenced to ensure that no errors occurred in PCR.

***lacZ* Reporter Constructs**

The p*ZRT1*-361 and p*ZRT1*-305 insertional plasmids (Gift of D. Eide) have the *lacZ* gene driven by the various truncations of the *ZRT1* promoter (-361 to +3 and -305 to +3). These plasmids were transformed into wild type (BY4742 and DY1457) backgrounds to obtain reporter strains. p*FET3*-*lacZ* episomal reporter plasmids, such as p*FET3*-398, p*FET3*-297, p*FET3*-263, p*FET3*-233 and p*FET3*-197 have a *lacZ* gene that is driven by different truncations of the *FET3* promoter (-398 to +3,-297 to +3, -263 to +3, -233 to +3, and -197 to +3, respectively (Gift of A. Dancis, University of Pennsylvania)). Several different reporters have been obtained that have the *lacZ* gene driven by a minimal *CYC1* promoter in which the native Upstream Activating Sequence has been replaced with fragments from various promoters. The p*ZRT1*-ZRE construct (pDg2) contains ZRE1 from the *ZRT1* promoter along with 8 base pairs of upstream and 10 base pairs of downstream flanking sequence (Gift of D. Eide). The construction of p*IZH1*-*ZRE* contains a similarly sized fragment containing the ZRE from the *IZH1* promoter (pDgIZH1ZRE). (The exact sequences of the inserted fragment are shown in figure 2-1C. p*CYC1*-FeRE (pFL-W) contains the following fragment of the *FET3* promoter containing the Fe^{3+} Response Element (FeRE, underlined): CTTCAAAAG<u>TGCACCC</u>ATTTGCAGGTGC.

β –Galactosidase and Ferroxidase Assays

Yeast transformations were performed by the litium acetate method to introduce plasmids into appropriate strains (Gietz and Woods, 2002). Yeast was grown for 24 hours in low zinc media (LZM) or low iron media (LIM) supplemented with varying concentrations of $ZnCl_2$ or $FeCl_3$. LZM or LIM is made zinc or iron deficient by 1 mM EDTA and is buffered at pH 4.0 with 20 mM sodium citrate. Galactose or raffinose (Acros #17629-30-0) was used as the carbon source. LZM or LIM contains normal concentrations of minerals except for $ZnCl_2$ or $FeCl_3$, which are varied from 1 μM to 1 mM. Cells grew to mid-log phase in LIM or LZM, where β-galactosidase (Lyons et al., 2004) or ferroxidase (De Silva et al., 1995) assays were performed following published procedures. β-Galactosidase assays measured the hydrolysis of 1-nitrophenyl-β-D-galactopyranoside (ACROS Organics #128820050) to 2-nitrophenol and monitored at 420 nm, which was measured at 20 minutes and quenched by sodium bicarbonate. β –Galactosidase activity is measured in Miller units and was calculated as follows: (A_{420} x 1,000) / min x mL of culture x A_{600}). Ferroxidase assays followed the disappearance of Fe^{2+}, from ferrous ammonium sulfate, which was measured at 5 minutes and quenched by ferrozine (ACROS Organics #410570050). The cells were centrifuged and the absorbance of the supernatant was monitored at 564 nm ($\epsilon = 0.0279\ \mu M^{-1}cm^{-1}$). The rate of Fe^{2+} oxidation in the absence of cells was subtracted to give the final value. Ferroxidase assay is measured in μmol Fe^{2+} oxidized per minute and was calculated as follows: (OD_{564} x 1 x 10^{-3} L) / (0.0279 $\mu M^{-1}cm^{-1}$ x min x OD_{600} x 1.5 x 10^8 cells). β –Galactosidase and ferroxidase activities were presented as "% of Control," which signifies activity expressed as percent of activity measured in the wild type strain carrying the empty vector control. All experiments include triplicate data points and each experiment was performed three times. Representative experiments are shown. All error bars represent ± standard deviation within a single experiment.

Ferrozine Plates

Iron-limited plates were made by adding 20 μM $FeCl_3$ to plates containing synthetic media, galactose, and 1 mM of the iron-specific chelator, ferrozine.

Protein Isolation

Frozen pellets were resuspended in 4 mL MIB buffer (0.6 M mannitol, 20 mM HEPES, 1 mM EDTA) to which the following inhibitors were added immediately: 1 mM PMSF and 10 μg/mL protease inhibitor cocktail (for fungal and yeast extracts Sigma #P8215). Cells were transferred to low binding microcentrifuge tubes and disrupted by adding acid washed beads and vortexing five times for 1 minute, resting 1 minute on ice between each disruption. Cells were then centrifuged at 3,000 rpm at 4ºC for ten minutes and the supernatant was collected. The supernatant was spun at 45,000 rpm for 90 minutes at 4ºC. The pellet was collected and suspended in MIB buffer with 33% glycerol and frozen at -80ºC.

Gel Electrophoresis

Samples were diluted to 1.0 mg/mL and combined with equal amounts of 2x SBB buffer (3% bromophenol blue, 0.2 M Tris-HCl pH 6.8, 20% glycerol, 10% β-mercaptoethanol, 2% SDS). Equal amounts of total protein were loaded on a 10% SDS-PAGE gel and run at 120 volts for two hours. The gel was stained with Coomassie Blue stain (0.02% Coomassie Blue R-250, 5% glacial acetic acid, 40% methanol) for 1 hour and counterstained with De-stain solution (10% glacial acetic acid, 5% methanol) overnight.

Western Blot

Proteins were transferred onto Hybond P (PVDP) membrane (American Biosciences #RPN303F) with electrophoresis transfer for one hour in 10% methanol, 1x Tris-Glycine, 0.1% SDS. The membrane was washed twice with 1x TBST (0.1% Tween 20) and then blocked with 5% milk in 1x TBST for one hour at room temperature. The membrane was incubated overnight

at 4ºC with the primary antibody (HA-probe rabbit polyclonal antibody (Open Biosystems #sc805), 1:500 dilution) in 1% bovine serum albumin (BSA) in 1x TBST, followed by washing the membrane three times with 1x TBST at room temperature. The secondary antibody (Goat anti-rabbit HRP conjugate (Open Biosystems #sc2004), 1:5,000 dilution) in 1% BSA in 1x TBST was added for one hour at room temperature followed by washing the membrane three times with 1x TBST at room temperature. The bands were detected with ECL Plus Western Blotting detection System (GE Healthcare #RPN2132).

Results

Effect of *IZH2* Overexpression on the *ZRT1* Promter

Previous studies showed that the p*ZRT1*-ZRE1 (also called pDg2) and *pIZH1*-ZRE reporters respond to the amount of zinc added to LZM. β-Galactosidase activity is fully induced when grown in LZM containing 1 μM zinc and repressed in LZM containing 1 mM zinc. This occurs due to reporters containing a ZRE, to which Zap1p must bind for transcriptional activation. Overexpression of the *IZH2* gene during zinc-deficiency results in repression of p*ZRT1*-ZRE. But the overexpression of two other membrane proteins, hZIP1, a human zinc uptake transporter that does not function in yeast (Gaither and Eide, 2001), or *ZRT1Δ2*, a non-functional mutant of yeast *ZRT1* (Gitan et al., 2003), does not effect p*ZRT1*-ZRE1 *lacZ* reporter. This suggests the effect of *IZH2* overexpression on p*ZRT1*-ZRE1 is not caused by aberent protein trafficking (Figure 2-2A). Also, *IZH2* overexpression did not repress the p*IZH1*-ZRE reporter, which contains a different ZRE (Figure 2-2B). This suggests that *IZH2* overexpression did not have a generalized effect on Zap1p activity.

We examined the effect of truncated p*ZRT1-lacZ* reporter constructs on β-galactosidase activity. A *ZRT1* promoter that contained 361 base pairs of the promoter was fused to a *lacZ* construct. This construct contains two known ZREs. ZRE1 was located at –318 to –308, while a

second functional ZRE is located between –203 to –193 (Figure 2-2D). The p*ZRT1*-361 *lacZ* construct responds reciprocally to the amount of zinc added to LZM, and *IZH2* overexpression repressed of activity of this reporter. A second construct that contained 305 base pairs of the *ZRT1* promoter was used, and was shown to be zinc responsive due to the second functional ZRE; however, overexpression of *IZH2* could not repress the *pZRT1*-305 *lacZ* construct (Figure 2-2E).

Effect of *IZH2* Overexpression on the *FET3* Promoter

In this paper, we also studied the effect of *IZH2* overexpression on a p*FET3-lacZ* reporter construct. The p*FET3-lacZ* reporter contains 398 base pairs of the *FET3* promoter and responds inversely to the amount of iron added to LIM. The increased activity of β-galactosidase, which is due to the transcription factor Aft1p binding to a **Fe**$^{3+}$ **R**esponse **E**lement (FeRE) between –250 to –244 (Yamaguchi-Iwai et al., 1996), is repressed by *IZH2* overexpression (Figure 2-3A). Ferroxidase activity, the physiological function of Fet3p, also showed full activity during iron deficiency and was repressed by *IZH2* overexpression (Figure 2-3B). We also demonstrated that repression of p*FET3-lacZ* by *IZH2* overexpression was incrementally alleviated by decreasing the galactose concentration, which decreases expression of genes driven by the *GAL1* promoter (Figure 2-3C). Agar plates made iron-deficient by the addition of ferrozine were used to compare growth of wild type cells carrying an empty vector or *IZH2* overexpressor. Wild type cells with the empty vector were capable of growth; however, the cells containing the *IZH2* overexpressor did not exhibit growth (Figure 2-3D).

We examined the effect of truncated p*FET3-lacZ* reporter constructs on β-galactosidase activity (Figure 2-3E). The p*FET3-lacZ* reporters containing 297 or 263 base pairs of the *FET3* promoter were inducible in iron-deficient LIM because both constructs contained functional FeRE, but were unable to respond to *IZH2* overexpression. Also, the p*FET3*-FeRE reporter,

which contains only the FeRE from the *FET3* promoter inserted into the minimal *CYC1* promoter, responds in an iron-dependent manner, but is not induced by *IZH2* overexpression. Both p*FET3*-233 and p*FET3*-197 constructs were not inducible when grown in iron-deficient LIM (Figure 2-3F).

Izh2p was expressed to ensure functionality; therefore we tagged Izh2p with 3x-HA epitope at the C-terminus. The overexpression of *IZH2*-HA was able to repress p*FET3*-398 similarly to *IZH2* overexpression (Figure 2-4A). Furthermore, we showed by Western blot that *IZH2*-HA construct is galactose inducible, but not glucose inducible, when grown in iron-deficient LIM (Figure 2-4B).

Msn2p and Msn4p Activates *FET3*

We were also interested in identifying activators and repressors of p*FET3-lacZ*. *FET3* has been shown to be induced during the diauxic shift in iron replete LIM (Haurie et al., 2003). Since Msn2p and Msn4p are known to activate gene transcription during this growth phase, we tested their role on *FET3* regulation. In *msn2Δ* and *msn4* mutant strains, ferroxidase activity is constitutively repressed. We also demonstrated that ferroxidase activity was repressed in an *msn2Δmsn4Δ* mutant strain compared to the wild type control. To examine Msn2p/Msn4p role during stress response, Marilee Weaver used the BY4742 wild type strain containing the p*CYC*-STRE *lacZ* reporter and found its activity decreased when it entered stationary phase. Further examination showed that the p*CYC*-STRE *lacZ* reporter was repressed when *IZH2* was overexpressed in early log phase (OD_{600} = 0.04), but not in mid-log phase (OD_{600} = 1.60). In addition, we examined the effect of Msn2p on the *FET3* promoter. To examine the role of Msn2p, we utilized two p*FET3-lacZ* constructs one containing 398 base pairs and the other 297

base pairs. In iron deficient LIM, results showed p*FET3*-298 was activated in the *msn2Δ* mutant strain, while p*FET3*-398 was constitutively repressed.

Nrg1p and Nrg2p Represses *FET3*

Another scenario in the regulation of *FET3* could be the activation of a repressor. Studies from *Candida albicans* have found a CaNrg1p transcriptional repressor may regulate iron uptake genes (Murad et al., 2001). Therefore, we decided to examine the role of *S. cerevisiae* CaNrg1p homologues, Nrg1p and Nrg2p, in the signaling of *FET3*. Overexpression of *IZH2* in an *nrg1Δ* and *nrg2Δ* mutant strain resulted in complete loss of repression of p*FET3-lacZ* activity in iron-deficient LIM. Also, the loss of *NRG1* and *NRG2* genes causes an increase in basal p*FET3-lacZ* activity. *IZH2* overexpression in an *nrg1Δnrg2Δ* mutant strain also led to a loss of repression of ferroxidase activity. In addition, we obtained a p*FLO11-lacZ* reporter that is known to be negatively regulated by Nrg1p and Nrg2p (van Dyk et al., 2005). *IZH2* overexpression was able to repress p*FLO11-lacZ* activity in iron-deficient LIM. Furthermore, we obtained a *GAL1*-driven TAP-tagged Nrg1p and Nrg2p constructs and found that overexpression of Nrg2p-TAP repressed p*FET3-lacZ* in a wild type strain when grown in iron-deficient LIM. On the other hand, the Nrg1p-TAP overexpressor could not repress the activity of p*FET3-lacZ*. In an *nrg1Δnrg2Δ* mutant strain, the overexpression of Nrg2p-TAP did not exhibit repression of ferroxidase activity in iron-deficient LIM.

The constitutive repression of ferroxidase activity in an *msn2Δmsn4Δ* mutant strain is alleviated if Nrg1p and Nrg2p both are also knocked-out. Furthermore, overexpression of *IZH2* in the *nrg1Δnrg2Δmsn2Δmsn4Δ* mutant strain exhibited no effect on ferroxidase activity.

Discussion

The Izh2p encodes a membrane protein that belongs to a large and ubiquitous family found in prokaryotes and eukaryotes. This family has been named the PAQR (Progestin and AdipoQ Receptor) family because it contains human adiponectin (Yamauchi et al., 2003) and membrane progestin receptors (Zhu et al., 2003a). This family is noted for containing at least seven transmembrane domains and four highly conserved motifs. Also, they have been found to be involved in metal regulation (Lyons et al., 2004).

Repression of *FET3* and *ZRT1* Involves the CCCTC Regulatory Element

In *S. cerevisiae*, the high affinity zinc uptake system is controlled by the transcription factor Zap1p by binding to zinc response elements, which induce genes during zinc deficiency such as Zrt1p (Zhao and Eide, 1996). Zrt1p activity was measured by the p*ZRT1*-ZRE1 reporter in which a fragment of the *ZRT1* promoter containing a known ZRE was inserted into the minimal *CYC1* promoter. This reporter construct is inducible by zinc deficiency in a Zap1p inducible manner. However, we showed that overexpression of the Izh2p repressed activity of the promoter construct regardless of nutritional zinc status. We verified that the negative effect of Izh2p on *lacZ* was not an artifact of our experimental design. First, previous studies from our group showing *IZH2* overexpression using an estradiol-inducible system reduces expression of the promoter driven by ZRE1 from *ZRT1* during zinc limitation, thus demonstrating the effect was not due to our method of induction. Second, overexpression of two non-functional membrane proteins could not exhibit repression of our *lacZ* reporter construct, indicating that repression was not due to generalized effect of protein folding or trafficking by overexpression of a membrane protein (Lyons et al., 2004). Also, previous studies that exhibited *IZH2* overepression did not affect cellular zinc accumulation (Lyons et al., 2000).

Since Zap1p is only known to be regulated by cellular zinc, we propose that the Izh2p does not target Zap1p. We verified this theory by working with another Zap1p-dependent reporter, p*IZH1*-ZRE. But, when Izh2p was overexpressed, repression of this reporter construct was not seen, even though the reporter contained a functional ZRE inserted into the same minimal *CYC1* promter, thus confirming *IZH2* overexpression does not have a generalized effect on Zap1p activity.

Functional ZREs are contained in both p*ZRT1*-ZRE1 and p*IZH1*-ZRE and are dependent on Zap1p (Figure 2-1C). But, the exact sequence of ZRE and flanking bases are slightly different. Thus, we postulated that ZRE1 in the *ZRT1* promoter contains an overlapping repressing element that is repressed by overexpression of Izh2p. To test whether *IZH2* could regulate the full *ZRT1* promoter, we utilized a series of p*ZRT1* reporter constructs, one containing 361 nucleotides of the *ZRT1* promoter and two ZREs, ZRE1 and ZRE2, while the other reporter construct contained 305 nucleotides of the *ZRT1* promoter and one ZRE. ZRE2 contains a sequence ACCTTTAAGGT located between -203 and -191, while ZRE1 contains a sequence of ACCCTCAAGGT located between -318 to -309. The p*ZRT1*-361 and p*ZRT1*-305 are both induced during zinc deficiency; however, induction of p*ZRT1*-361 is partially repressed by *IZH2* overexpression, but not p*ZRT1*-305. This data suggests that the proposed repressive element can be found between -361 and -305 of the *ZRT1* promoter and that it most likely overlaps the 29 base pairs surrounding ZRE1.

In investigating the specificity of *IZH2*-dependent repression, we discovered that the *IZH2* gene also affected the high affinity iron uptake system. This system is controlled by the transcription factor Aft1p (Rutherford, et al., 2004), which senses cellular iron levels and induces expression of two genes, *FET3* and *FTR1*, whose genes function in tandem to form an oxidase-

permease system that takes iron into the cell during deficiency. In our experiments, p*FET3-lacZ* reporter construct was used to measure *FET3* activity. First, we demonstrated that overexpression of the *IZH*2 repressed the p*FET3-lacZ* reporter. To verify that this was not an artifact of the *lacZ* construct, we could have performed one of three techniques to answer this question: Northern blot, RT-PCR, or activity of Fet3p. We decided to pursue Fet3p activity because it would show functionality, and found that *IZH2* overexpression decreased Fet3p enzymatic activity in low iron media, confirming an actual loss in the Fet3p phenotype. The levels of repression of p*FET3-lacZ* and ferroxidase activity were similar to repression caused by iron repletion, suggesting that the loss in ferroxidase activity is caused by decreased gene expression.

We also showed that repression by p*FET3-lacZ* was not an artifact of our experimental design. First, *IZH2* overexpression, which is driven by a *GAL1* promoter, exhibited an inability to repress p*FET3-lacZ* when the medium was incrimentally switched from galactose to raffinose, demonstrating repression was due to *IZH2* overexpression. We decreased the percentage of galactose, which ramps down the expression of the Izh2p, thereby preventing its effect on *FET3*. Second, we showed cell viability in iron-limited LIM is decreased by overexpression of Izh2p, thus confirming that the Izh2p effect is not a physiological irrelevant phenomenon.

To identify the repressing element in the *FET3* promoter, we obtained a series of p*FET3-lacZ* reporter constructs, p*FET3*-398, p*FET3*-297, p*FET3*-263, and p*FET3*-FeRE, which contain the consensus FeRE sequence TGCACCC located between -252 to -245. All p*FET3-lacZ* constructs responded to cellular iron levels in an Aft1p-dependent manner (Yamaguchi-Iwai et al., 1996); however, only p*FET3*-398 exhibited Izh2p-dependent repression in iron-defecient LIM when *IZH2* was overexpressed. Therefore, we predict the repressing element is between -

398 and -297. When we look closer at the regions surrounding ZRE1 of the *ZRT1* promoter and the 100 base pair fragment of the *FET3* promoter, which led us to predict the repressing element is a CCCTC motif located at -316 to -312 in the *FET3* promoter. This same motif is found overlapping ZRE1 in the *ZRT1* promoter at -317 to -313.

Nrg1p/Nrg2p and Msn2p/Msn4p Regulate *FET3*

Two possibilities exist in which the Izh2p may function to repress *FET3*. One is the inactivation of a co-activator. But, at this time, Aft1p is the only known activator of *FET3* induction. However, we wanted to know if *IZH2* overexpression can affect Aft1p. We tested this theory by using a series of p*FET3-lacZ* constructs, one containing the 398 base pairs and other containing 30 base pairs, located between -263 and -234 of the *FET3* promoter, but still containing the Fe^{3+} Response Element (FeRE). Therefore, the p*FET3*-FeRE can still respond to iron deficiency by Aft1p. We demonstrated *IZH2* overexpression could repress the p*FET3*-398 reporter, but not the p*FET3*-FeRE reporter. Thus *IZH2* overexpression modulates *FET3* activity in an iron-independent manner.

Is there there another activator of *FET3*? Haurie, et al showed that *FET3* is also induced during the diauxic shift (Haurie et al., 2003). Since the stress responsive transcription factors Msn2p and Msn4p are known to activate gene transcription during the diauxic shift (Shenhar and Kassir, 2001), we examined their role in regulation of *FET3*. First, we demonstrated that Msn2p and Msn4p are essential for induction of *FET3* and that Msn2p/Msn4p may be negatively regulated by *IZH2* overexpression. Second, Marilee Weaver showed *IZH2* overexpression repressed a stress responsive reporter that is activated by Msn2p and Msn4p. Therefore, we propose Izh2p represses *FET3* by inactivating the repressors, Msn2p and Msn4p.

In addition, we were interested in whether the Msn2p/Msn4p effected *FET3* regulation by binding to a specific sequence in the *FET3* promoter. To test this theory, we used two p*FET3*-

lacZ constructs in an *msn2Δ* strain; one containing 398 base pairs and the other 297 base pairs. We demonstrated that p*FET3*-398 is constitutively repressed in the *msn2Δ*, but p*FET3*-297 responds normally in low iron media. Therefore, *FET3* activation by Msn2p mediated by an element located between 398 and 297 base pairs in the *FET3* promoter.

Nrg1p and Nrg2p Negatively Regulates *FET3*

A second mechanism of *FET3* repression may involve the Izh2p activating a repressor. This led us to a study performed in *Candida albicans*, showing that strains missing the CaNrg1p transcription factor, exhibited increased expression of high-affinity iron uptake genes (Murad et al., 2001). Also, we found that CaNrg1p has two homologues in *S. cerevisiae* named Nrg1p and Nrg2p. Deletion of either *NRG1* or *NRG2* results in inability of *IZH2* overexpression to repress *FET3*. Subsequently, we demonstrate that *IZH2* overexpression represses *FLO11*, a gene that is negatively regulated by Nrg1p and Nrg2p. Furthermore, repression of *FET3* was also exhibited by Nrg2p overexpression. Repression by Nrg1p/Nrg2p is proposed to occur by binding to a CCCTC consensus sequence in the promoters of target genes (Park et al, 1999). This consensus sequence can be found in the *FET3* promoter between -316 to -312.

Msn2p/Msn4p and Nrg1p/Nrg2p are Epistatic

Our data demonstrates that *FET3* is positively affected by Msn2p/Msn4p and negatively affected by Nrg1p/Nrg2p. Also, it is proposed that Nrg1p and Nrg2p bind to the consensus sequence CCCTC, which is similar to the CCCCT that serves as a binding site for Msn2p and Msn4p (Park et al, 1999). The similarity between CCCTC and CCCCT consensus sequences make it possible that Nrg1p/Nrg2p and Msn2p/Msn4p bind to the same consensus sequence. Intriguingly, this idea is bolstered by a recent study suggesting Nrg1p/Nrg2p and Msn2p/Msn4p compete for binding to the same consensus sequence in stress-responsive promoters (Vyas et al., 2005). Our data demonstrate that constitutive *FET3* repression seen in the *msn2Δmsn4Δ* mutant

strain is alleviated by the additional deletion of *NRG1* and *NRG2* in the *msn2Δmsn4Δnrg1Δnrg2Δ* mutant strain. This result suggests that Nrg1p/Nrg2p is epistatic to Msn2p/Msn4p with respect to *FET3* expression. However, at this time, we can not determine if Nrg1p/Nrg2p or Msn2p/Msn4p act on *FET3* through cis-regulatory elements. Therefore, we propose Nrg1p/Nrg2p and Msn2p/Msn4p act competitively and antagonistically on *FET3* regulation and that Izh2p overexpression may shift this competition by activating Nrg1p/Nrg2p and inactivating Msn2p/Msn4p.

Aft1p Activation is not Affected by Mutant Strains

In addition to this study, we demonstrated none of the deletion mutant strains affected *FET3* response to the amount of iron added to the media, which suggests that the mutant strains do not have a general affect on the ability of iron to inactivate the Aft1p iron-responsive transcriptional activator. Therefore, this response allows us to measure *IZH2* overexpression in these mutant strains.

Summation

Our findings support two conclusions. First, Izh2p activates a signal transduction pathway that represses both *ZRT1* and *FET3* expression. Second, Izh2p affects iron homeostasis via the Nrg1p/Nrg2p and Msn2p/Msn4p. Future experiments will focus on investigating the physical binding of NRG to the promoters and putative binding sites in question.

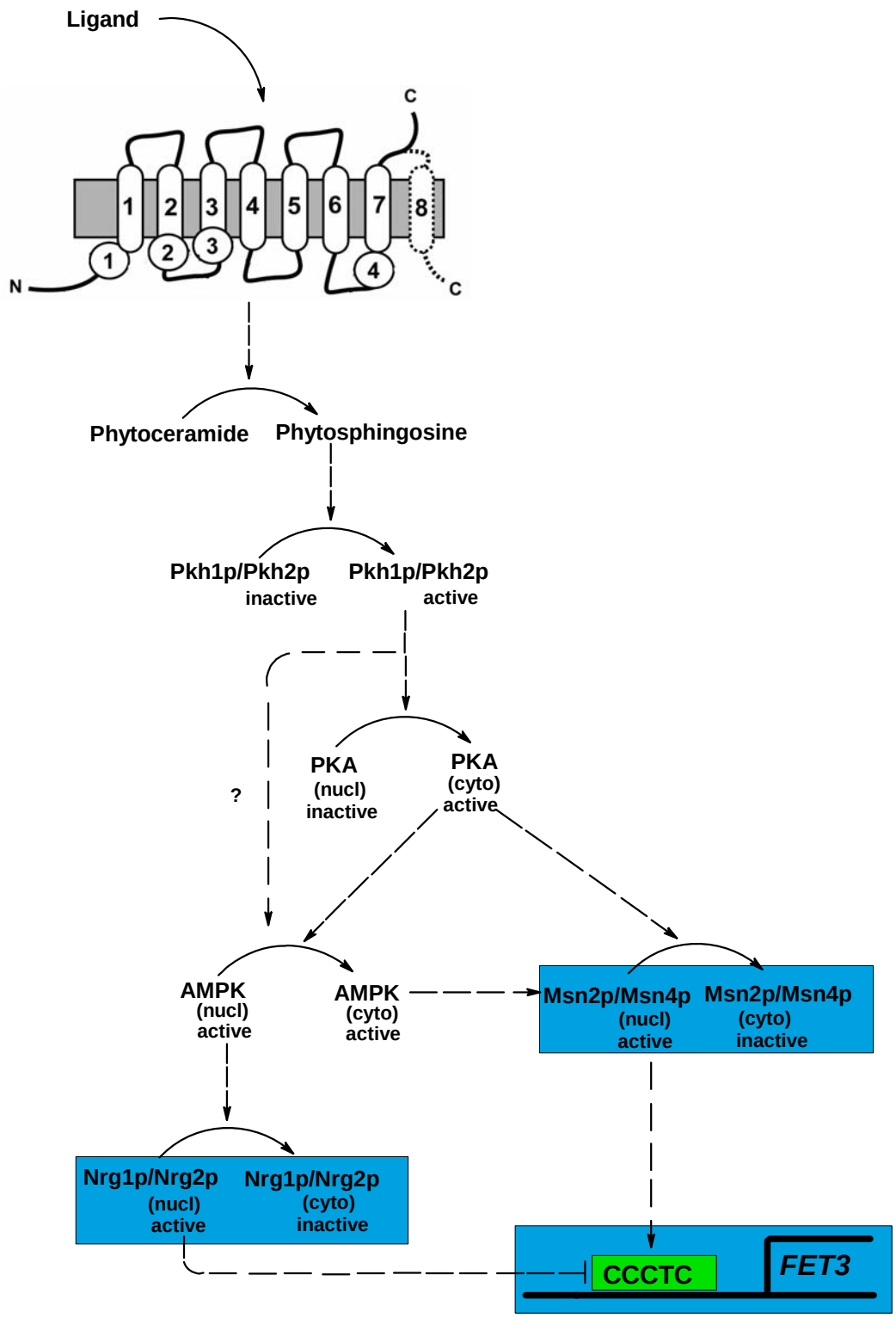


Figure 2-1. A model for the mechanism of *FET3* by *IZH2* overexpression. Solid arrows indicate protein activation or localization of protein. Broken arrows indicate protein interaction on localization and activation. Cyto = protein is cytoplamic. Nucl = protein is nuclear. Shaded blue portions represent areas studied in chapter.

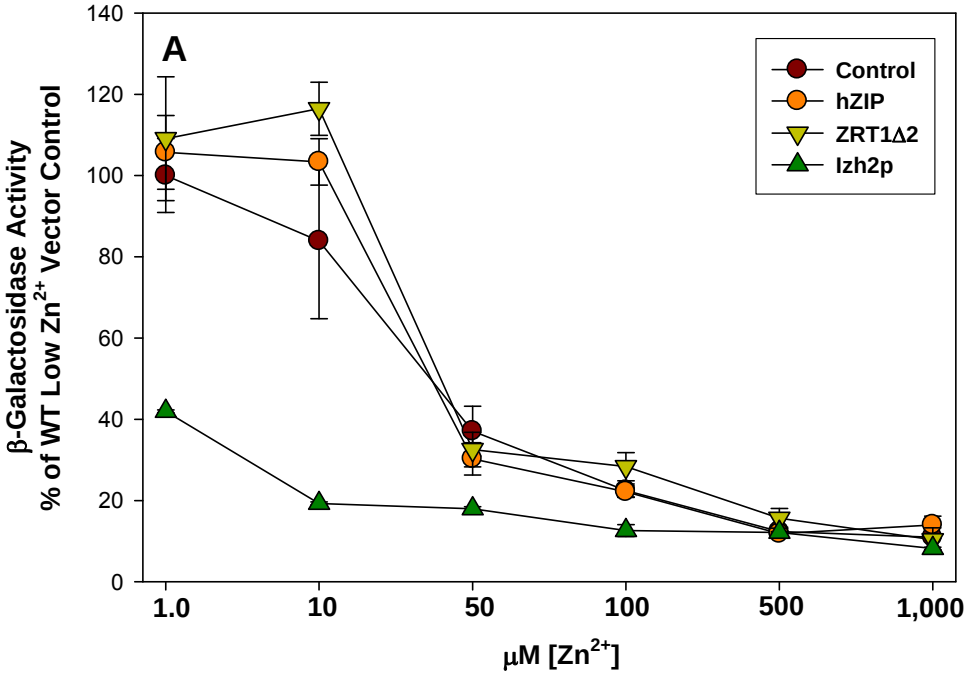
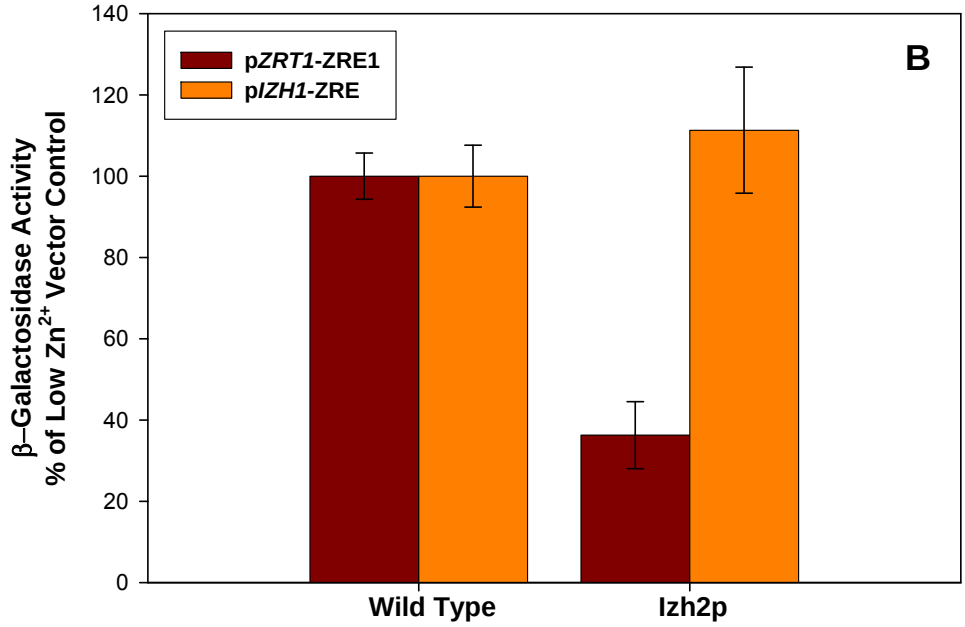


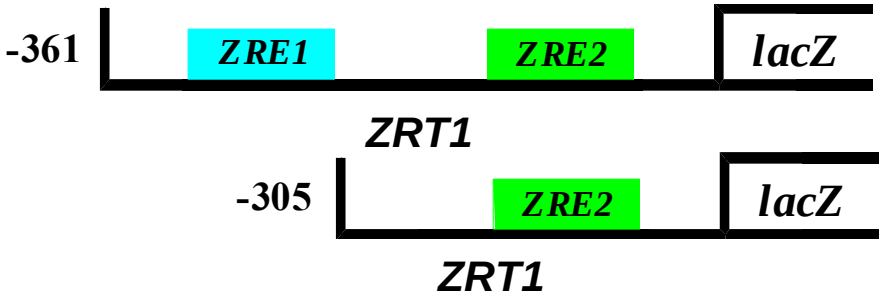
Figure 2-2. Repression of *ZRT1* by *IZH2*-overexpression. BY4742 wild type strain is used in panels A and B, while DY1457 wild type strain was used in panel E. β-Galactosidase activities are shown as a percentage of fully induced activity in wild type strain carrying an empty control vector grown in iron-deficient LIM. The error bars represent ± 1 standard deviation for experiments performed in triplicate. (A) β-Galactosidase activity of yeast strain carrying the p*ZRT1*-ZRE2 and the *IZH2* overexpressors individually. Cells are grown in LZM/galactose supplemented with 1 to 1,000 μM Zn^{2+}. (B) β-Galactosidase activity of yeast strain carrying the p*IZH1*-ZRE and the *IZH* overexpressors individually. Cells are grown in LZM/galactose supplemented with 1 and 1,000 μM Zn^{2+}. (C) Sequences of p*ZRT1*-ZRE1 and p*IZH1*-ZRE inserted into the minimal *CYC1* promoter. CCCTC is the putative binding site overlapping p*ZRT*-ZRE1. (D) Picture of *ZRT1* plasmid constructs: p*ZRT1*-361 and p*ZRT1*-305. Numbers represent nucleotides in *ZRT1* promoter. (E) β-Galactosidase activity of yeast strain carrying the p*ZRT1*-361 or p*ZRT1*-305 and the *IZH2* overexpressor. Cells are grown in LZM/galactose supplemented with 1 and 1,000 μM Zn^{2+}.



C

p*ZRT1*-ZRE1 CCAAAGATACCCTCAAGGTTCTCATCTGT
p*IZH1*-ZRE ATCATGCAACCTTTAGGGTCCAAGCCCTT

D



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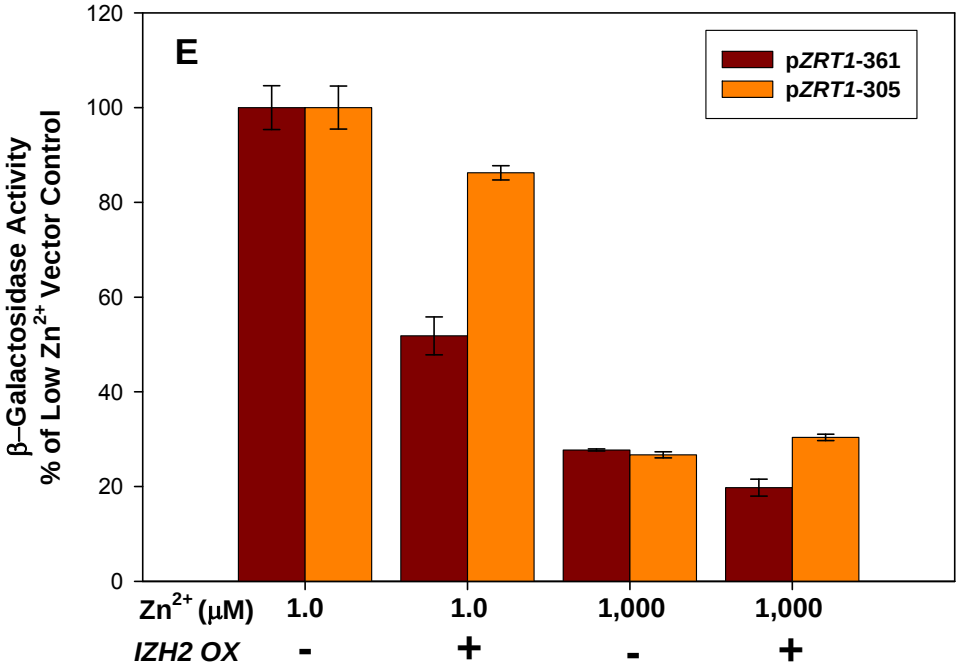

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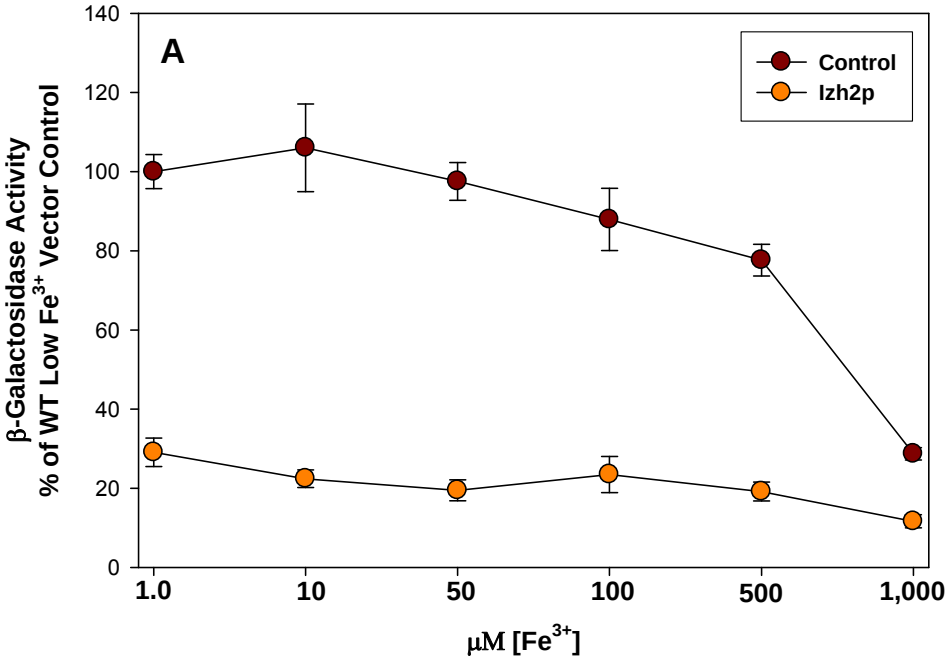
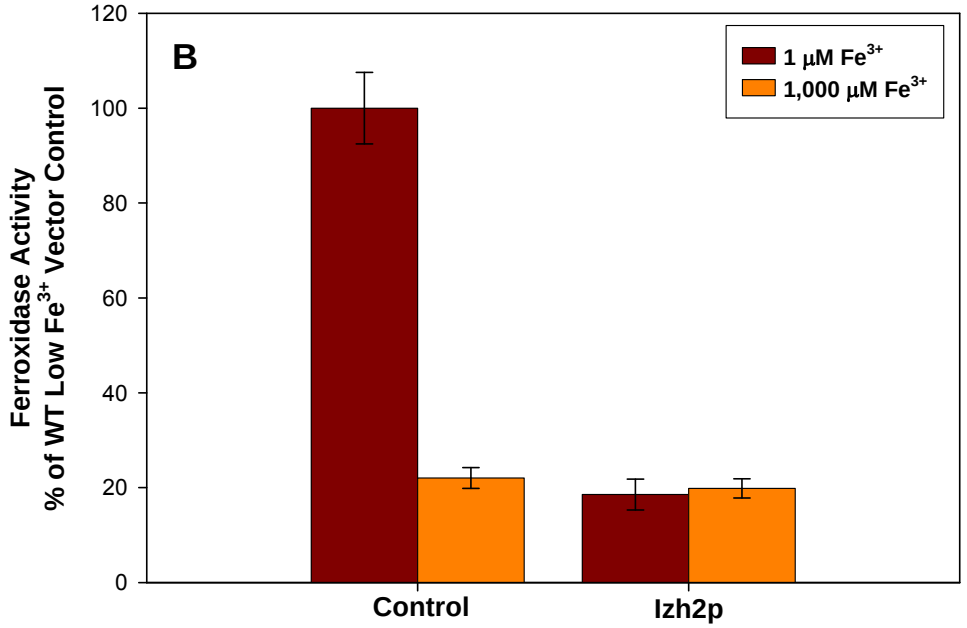
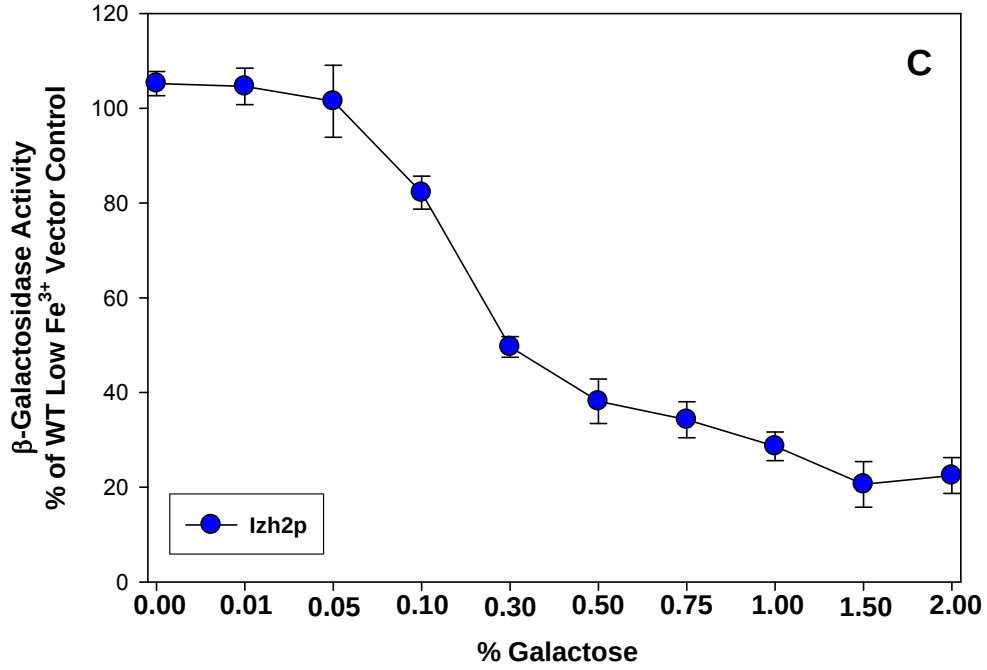


Figure 2-3. Repression of *FET3* by *IZH2*-overexpression. BY4742 wild type strain is used in all panels. β-Galactosidase (Panels A, C, F, and G) or ferroxidase (Panel B) activities are shown as a percentage of fully induced activity in wild type strain carrying an empty control vector grown in iron-deficient LIM. The error bars represent ± 1 standard deviation for experiments performed in triplicate. (A) β-Galactosidase activity of yeast strain carrying the p*FET3*-398 and the IZH overexpressors individually. Cells are grown in LIM/galactose supplemented with 1 to 1,000 μM Fe^{3+}. β-Galactosidase activity is induced at low concentrations of iron, but not at high concentrations of iron in the BY4742 wild type strain; however, *IZH2* overxpression represses p*FET3*-398 β-galactosidase activity. (B) Cell surface ferroxidase activity is induced by low iron in BY4742 wild type strain carrying an empty vector, but not in yeast carrying the IZH overexpressors. (C) Izh2p overexpression on p*FET3*-398 decreases as the % galactose in iron-deficient LIM is decreased. (D) Izh2p overexpression causes a growth defect in plates containing synthetic galactose media supplemented with 1 mM ferrozine and 20 μM Fe^{3+}. (E)Pictures of p*FET3*-lacZ constructs: p*FET3*-398, pFET3-297, p*FET3*-263, p*FET3*-233, p*FET3*-197 and p*CYC1*-FeRE. Numbers represent nucleotides in *FET3* promoter. (F) β-Galactosidase activity is induced in strains carrying p*FET3*-398, p*FET3*-297, p*FET3*-263, or p*CYC1*-FeRE in iron-deficient LIM. Izh2p overexpression in iron-deficient LIM represses only the p*FET3*-398. (G) β-Galactosidase activity is induced in strains carrying p*FET3*-398, but not with p*FET3*-233 or p*FET3*-197 in iron-deficient LIM. Izh2p overexpression in iron-deficient LIM represses only the p*FET3*-398.





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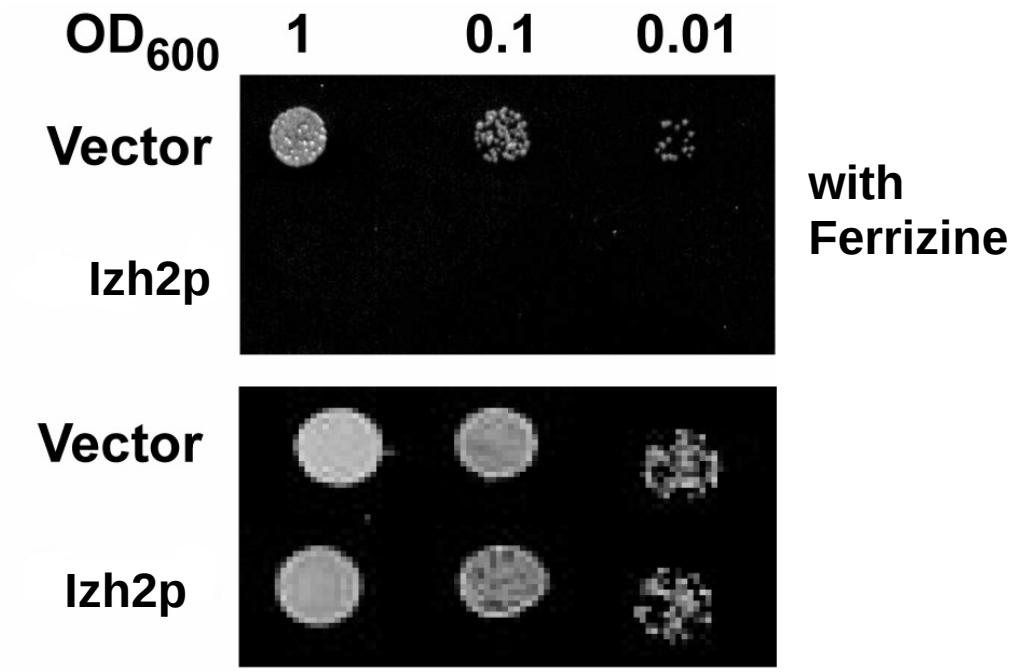
D



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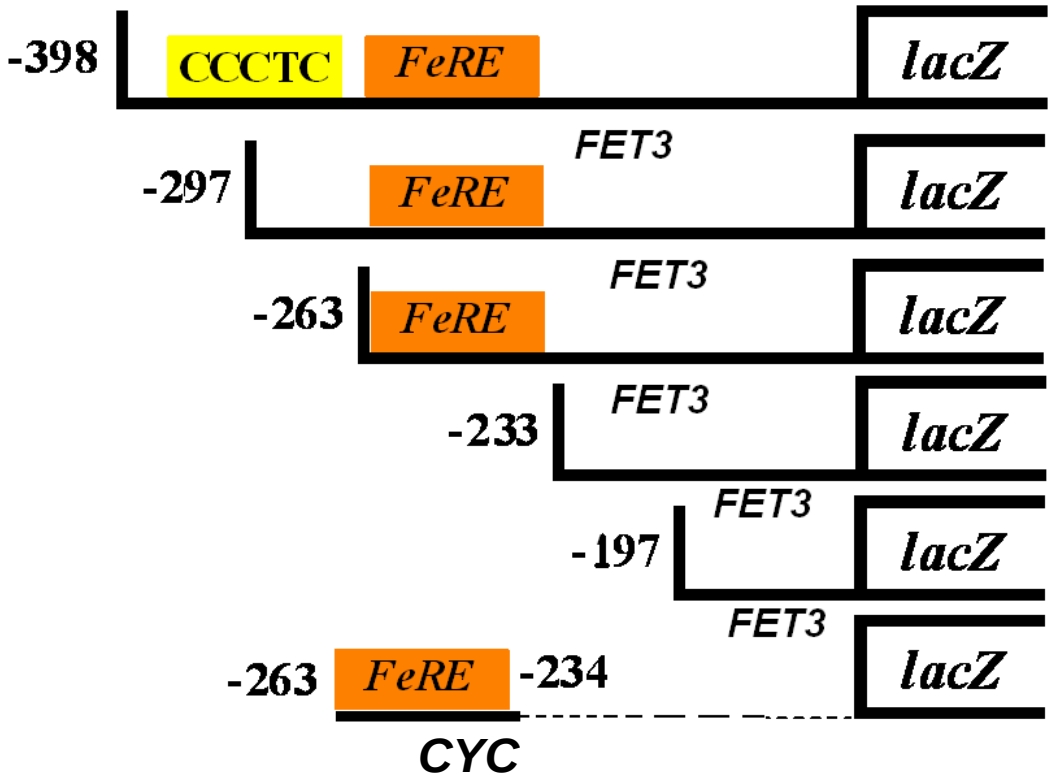

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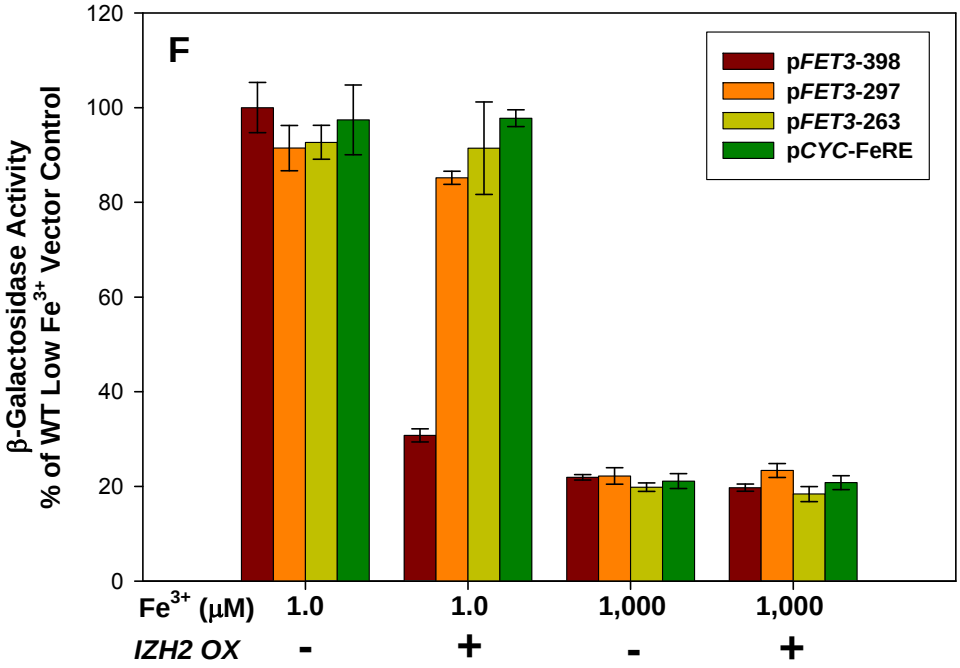

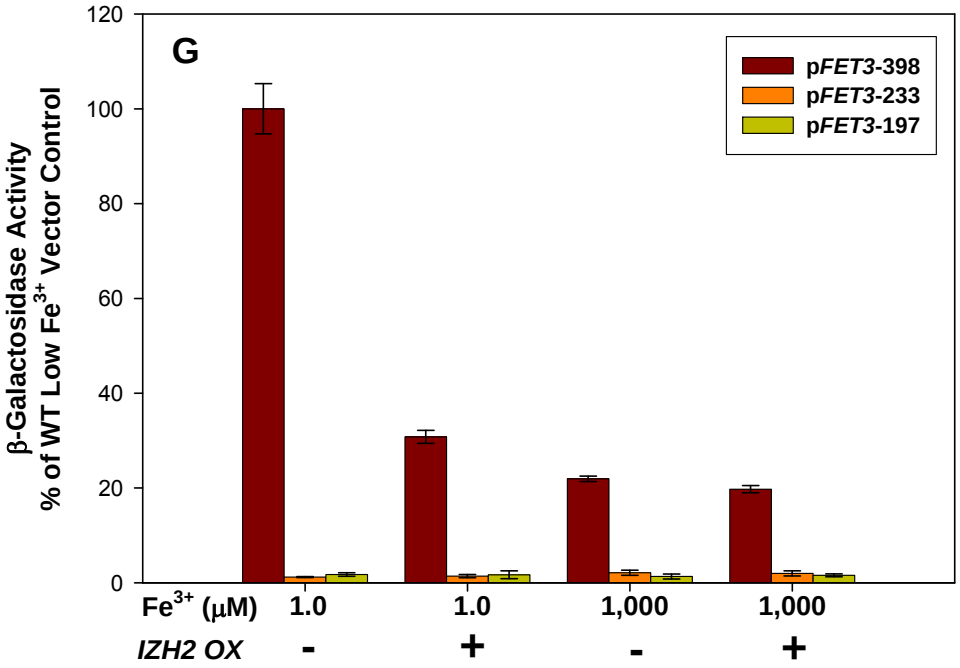

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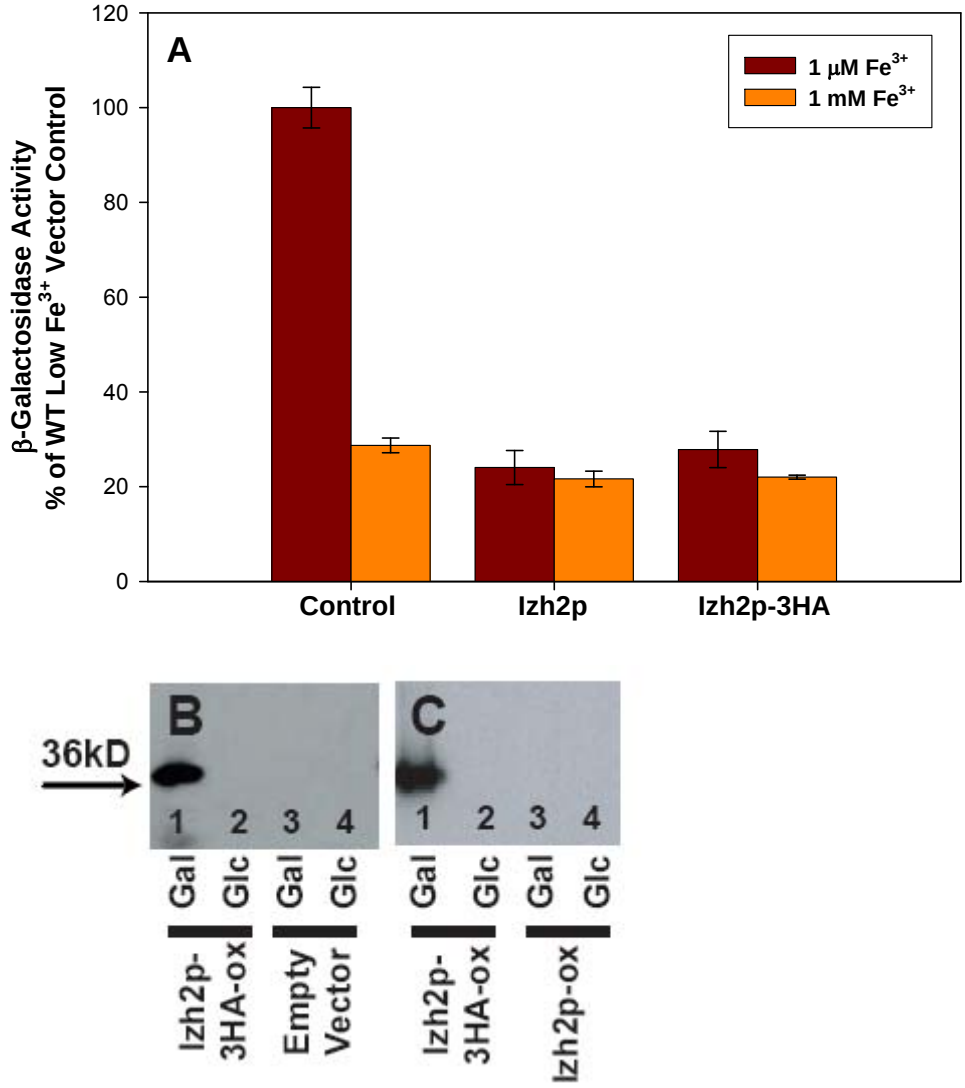


Figure 2-4. Izh2p-3HA expression. BY4742 wild type strain is used in panels A to C. β-Galactosidase activities are shown as a percentage of fully induced activity in wild type strain carrying an empty control vector grown in iron-deficient LIM. The error bars represent ± 1 standard deviation for experiments performed in triplicate. (A) β-Galactosidase activity of p*FET3*-398 is repressed in a strain carrying Izh2p or Izh2p-3HA overexpressors in iron-deficient LIM. (B) Western blot Izh2p-3HA tagged protein is induced in a strain grown in galactose but not in a strain grown in glucose. (C) Western blot showing Izh2p-3HA tagged protein is expressed in the strain carrying the Izh2p-3HA plasmid but not in the strain carrying the Izh2p plasmid.

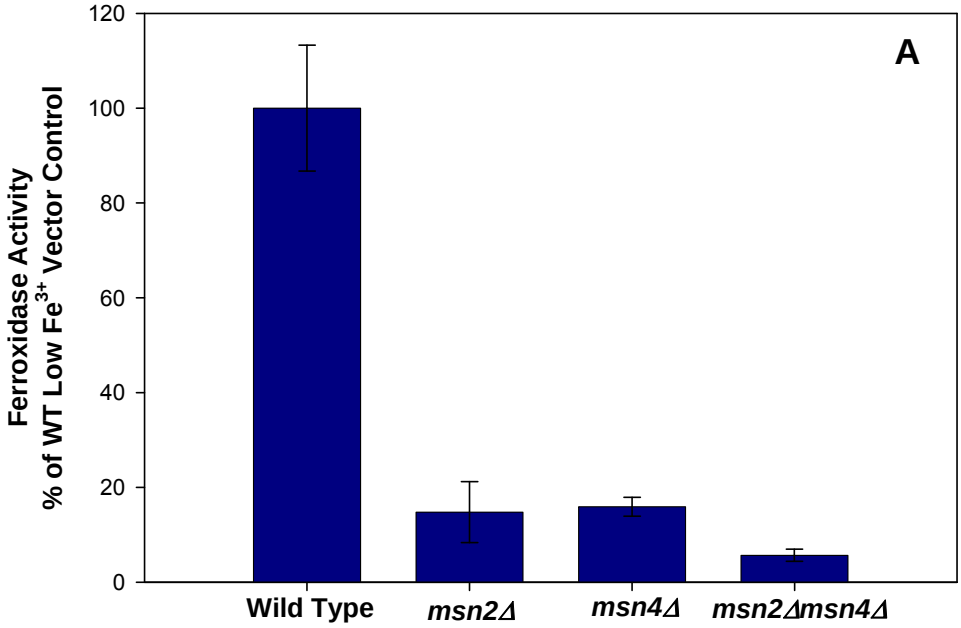
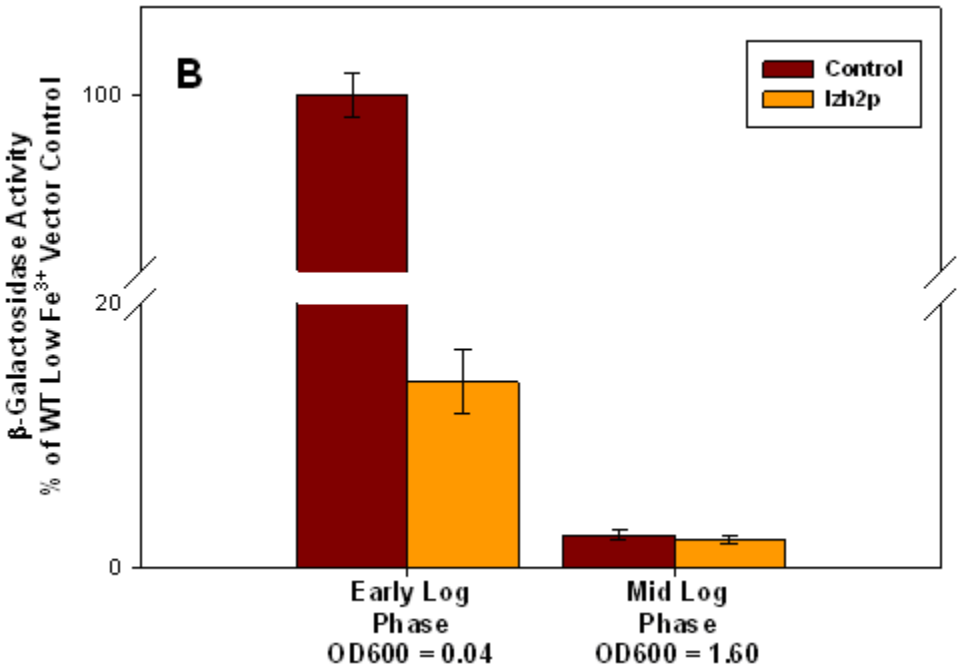
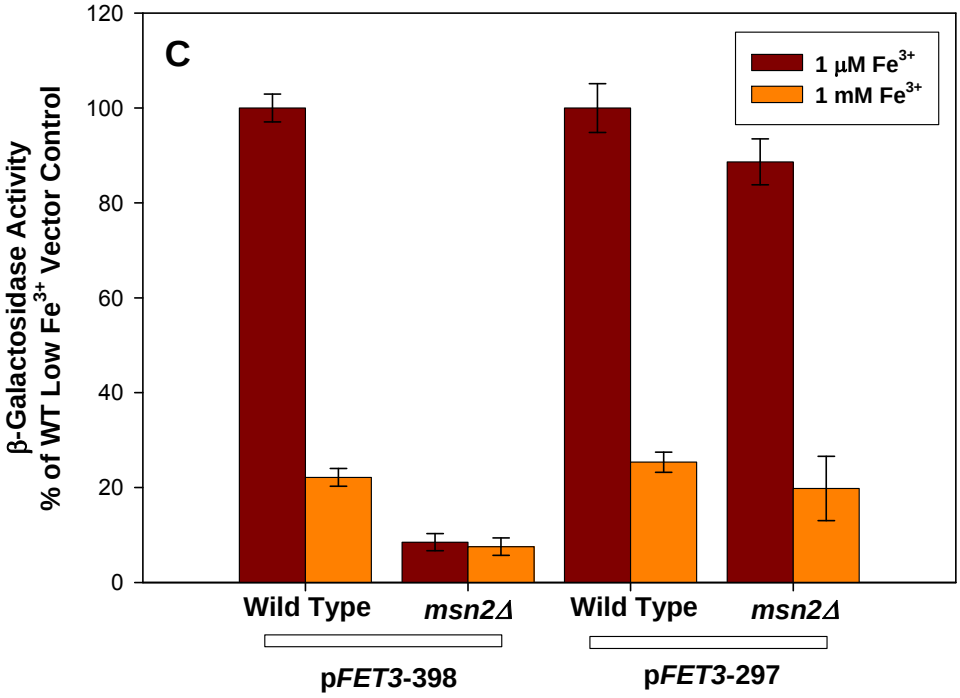


Figure 2-5. *MSN2* and *MSN4* positively regulate *FET3*. BY4742 wild type strain is used in panels A to C, while the wild type strain MCY5326 and *msn2Δmsn4Δ* mutant strain were used in panel B. *msn2Δ* and *msn4Δ* mutant strains were used in panel A. β-Galactosidase (Panels B and C) or ferroxidase (Panel A) activities are shown as a percentage of fully induced activity in wild type strain carrying an empty control vector grown in iron-deficient LIM. The error bars represent ± 1 standard deviation for experiments performed in triplicate. (A) Cell surface ferroxidase is repressed in *msn2Δ*, *msn4Δ,* and *msn2Δmsn4Δ* mutant strains. (B) p*CYC*-STRE *lacZ* activity is repressed when *IZH2* is overexpressed at OD_{600} 0.04, but not at OD_{600} 1.60. (C) p*FET3*-398 activity was constitutively repressed in the *msn2Δ* mutant strain, while p*FET3*-297 activity was induced.





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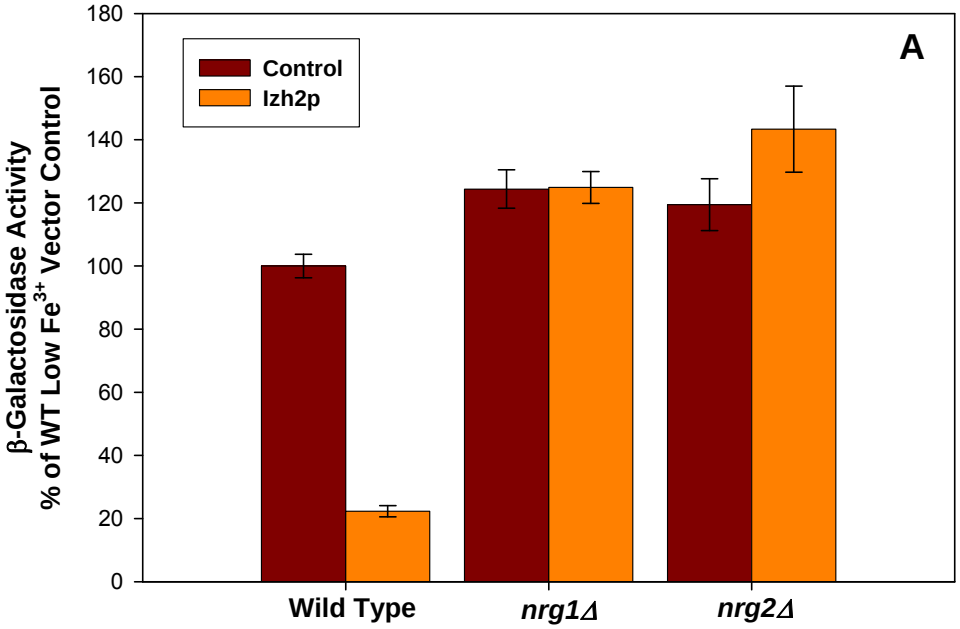
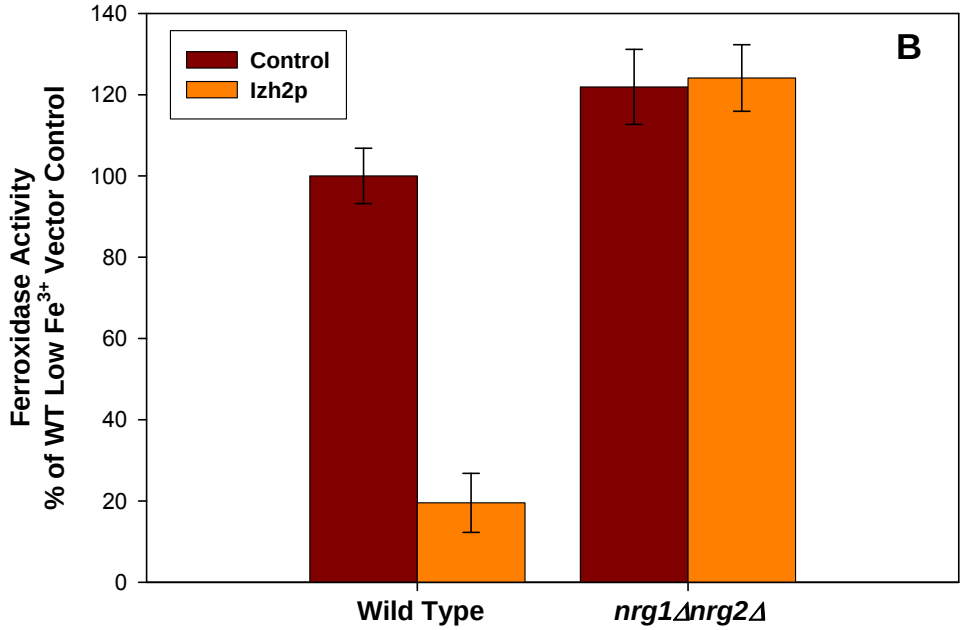
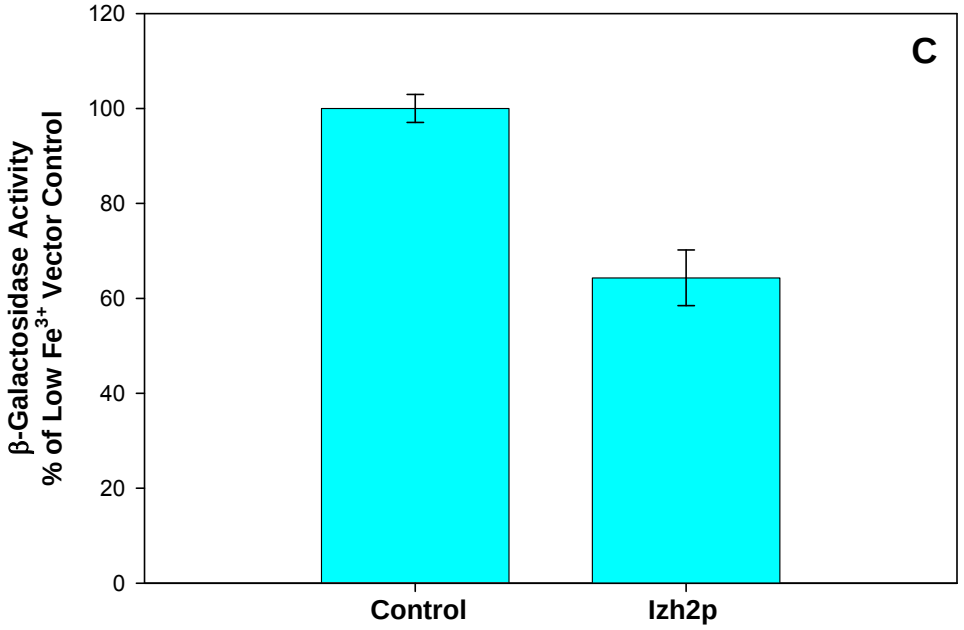


Figure 2-6. Nrg1p and Nrg2p are essential for repression. BY4742 wild type strain was used in panels A and D. MCY5326 wild type strain was used in panels B, E, and F. Σ1278b wild type is used in panel C. *nrg1Δ* (Panel A), *nrg2Δ* (Panel A), *nrg1Δnrg2Δ* (Panels B, E, and F), *msn2Δmsn4Δ* (Panel F), and *nrg1Δnrg2Δmsn2Δmsn4Δ* (Panel F) strains were also used. β-Galactosidase (Panels A, C and D) or ferroxidase (Panels B, E, and F) activities are shown as a percentage of fully induced activity in wild type strain carrying an empty control vector grown in iron-deficient LIM. The error bars represent ± 1 standard deviation for experiments performed in triplicate. (A) *IZH2* overexpression does not affect p*FET3-lacZ* activity in an *nrg1Δ* and *nrg2Δ* mutant strains. (B) Ferroxidase activity is not repressed by *IZH2* in an *nrg1Δnrg2Δ* mutant strain. (C) *IZH2* overexpression represses a *FLO11-lacZ* construct. (D) The TAP-tagged Nrg2p overexpressor, but not TAP-tagged Nrg1p overexpressor, repressed p*FET3-lacZ*. (E) Ferroxidase activity is not affected by TAP-tagged Nrg2p overexpression in an *nrg1Δnrg2Δ* mutant strains. (F) Ferroxidase was constititively repressed in *msn2Δmsn4Δ* mutant strain, partially repressed in an *nrg1Δnrg2Δmsn2Δmsn4Δ* mutant strain, and repression is alleviated in an *nrg1Δnrg2Δ* mutant strain. *IZH2* overexpression had no affect in these strains.





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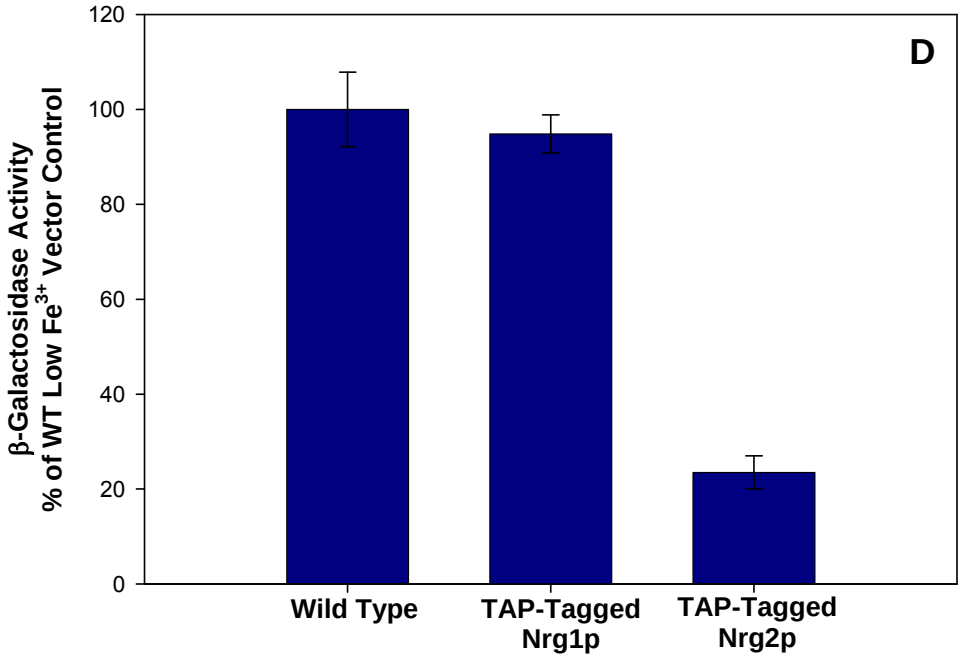

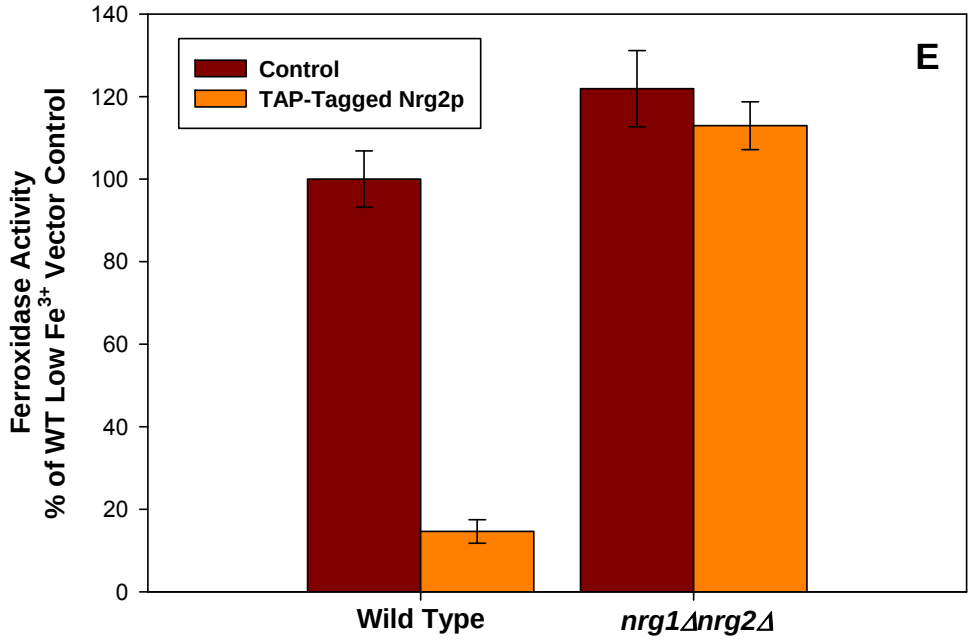

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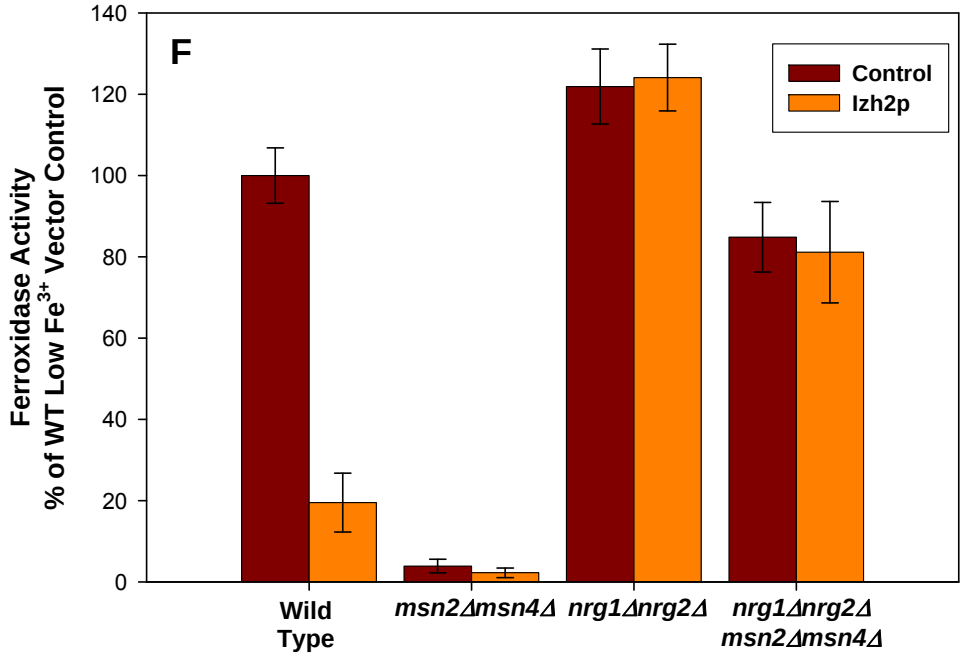

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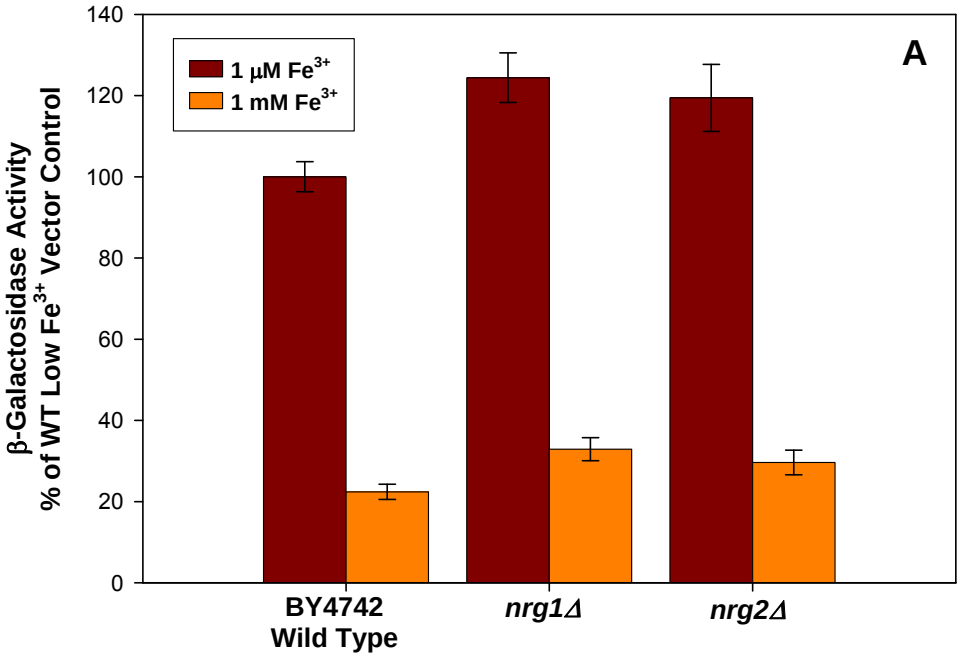
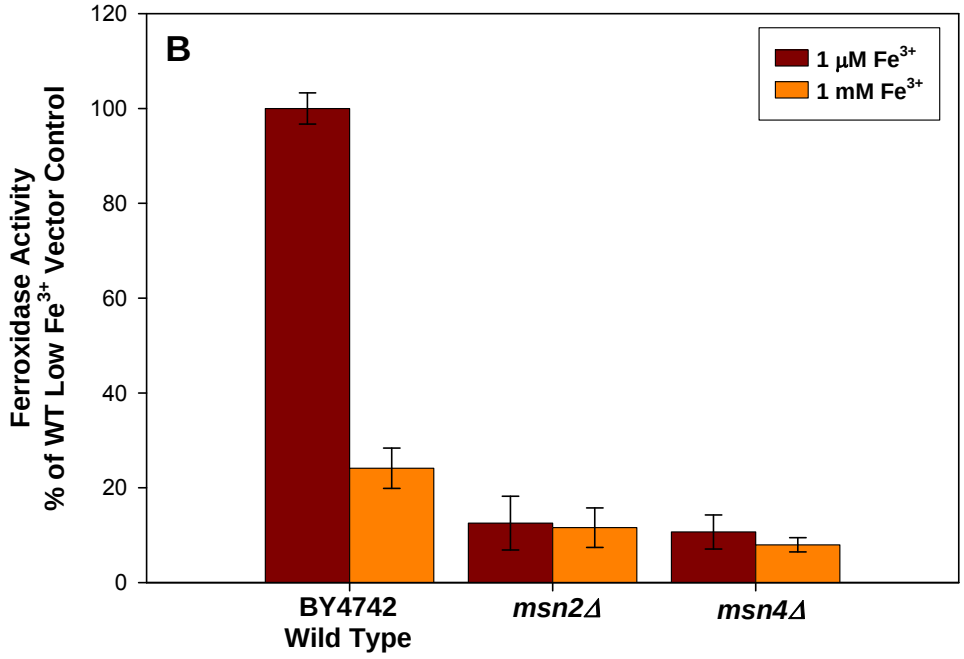


Figure 2-7. Aft1p responds normally to iron-defeciency in most mutant strains. BY4742 wild type strain was used in panels A and B. MCY5326 wild type strain was used in panel C. *nrg1Δ* (Panel A), *nrg2Δ* (Panel A), *msn2Δ* (Panel B), *msn4Δ* (Panel B), *nrg1Δnrg2Δ* (Panel C), *msn2Δmsn4Δ* (Panel C), and *nrg1Δnrg2Δmsn2Δmsn4Δ* (Panel C) mutant strains were also used. β-Galactosidase (Panel A) or ferroxidase (Panels B and C) activities are shown as a percentage of fully induced activity in wild type strain carrying an empty control vector grown in iron-deficient LIM. The error bars represent ± 1 standard deviation for experiments performed in triplicate. (A) β-Galactosidase activity is induced in iron-defeciency in the BY4742 wild type strain, and in strains lacking *NRG1 and NRG2*. (B) Ferroxidase activity is not induced in strains lacking *msn2Δ* and *msn4Δ*. (C) Ferroxidase activity is induced in iron-defeciency in the MCY5326 wild type strain, or in strains lacking *NRG1NRG2,* and *MSN2MSN4NRG1NRG2*. Ferroxidase activity is not induced in the strain lacking *MSN2MSN4*.



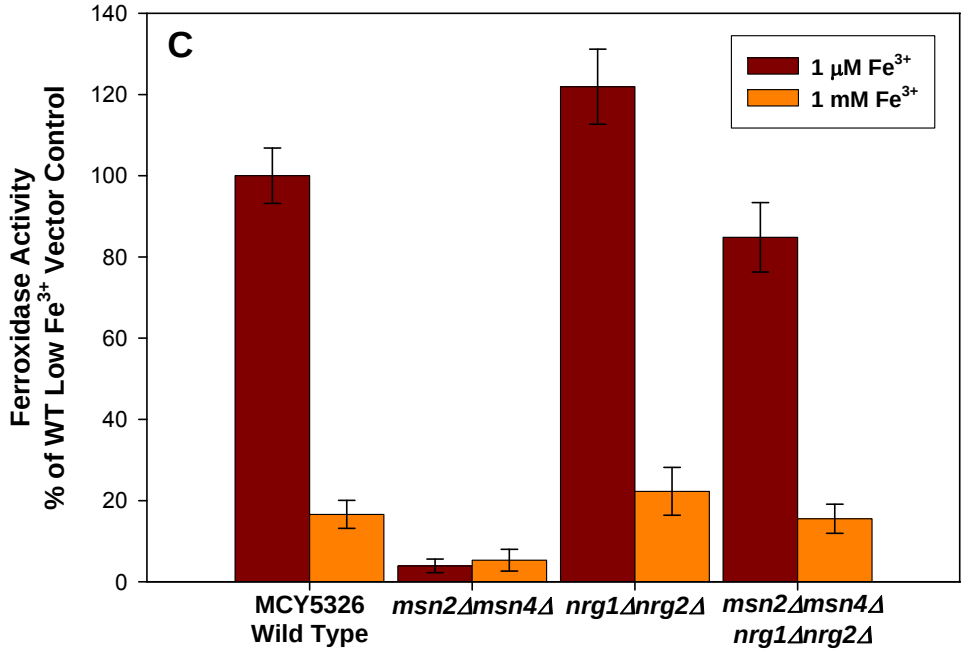


Figure 2-7 continued

CHAPTER 3
THE SIGNALING OF *FET3* REPRESSION BY *IZH2* OVEREXPRESSION

Introduction

Chapter 2 details experiments showing that *IZH2* overexpression represses *FET3* through the carbon source-dependent transcription repressors, Nrg1p and Nrg2p (Berkey et al., 2004) and the stress-responsive transcriptional activators, Msn2p and Msn4p (Smith et al., 1998). We also demonstrated that Nrg1p/Nrg2p and Msn2p/Msn4p likely compete for the same binding site on the *FET3* promoter that is essential for *IZH2*-dependent repression of *FET3*. In this chapter, we identified signaling proteins upstream of these transcription factors, which are required for *FET3* repression by *IZH2* overexpression. We show that *IZH2*-dependent repression of *FET3* requires protein kinase A (PKA), which inhibits Msn2p/Msn4p (Smith et al., 1998), and AMP-dependent kinase (AMPK), which inhibits Nrg1p/Nrg2p (Kuchin et al., 2002). Finally, we demonstrate that *IZH2*-dependent repression requires the sphingoid base sensing kinases Pkh1p and Pkh2p (Figure 3-1). Thus, these results suggest that *IZH2* overexpression activates PKA and inactivates AMPK that leads to repression of *FET3*.

Materials and Methods

Yeast Strains and Plasmids

The wild type (BY4742) strain and the *pkh1Δ*, *pkh2Δ*, *tpk1Δ*, *tpk2Δ*, *tpk3Δ*, *ras2Δ*, *tos3Δ*, *elm1Δ*, *sak1Δ*, *snf1Δ*, *snf4Δ*, *gal83Δ*, *sip1Δ*, and *sip2Δ* mutant strains were obtained from Euroscarf and their genotypes are listed in Supplementary Table 1. The p*FET3-lacZ* plasmid (Gift of D. Eide) has the promoter region of the p*FET3* gene fused to the *lacZ* open reading frame and is *URA3* selectable. *IZH2* was cloned from yeast genomic DNA into pRS316-*GAL1* (*LEU2*) via homologous recombination as previously described (Lyons et al., 2004). The primers used to

clone *IZH2* are listed in Supplementary Table 2. All clones were sequenced to ensure that no errors occurred during PCR.

β -Galactosidase and Ferroxidase Assays

β -Galactosidase and ferroxidase assays were performed as described in chapter 2 except for the addition of the following compounds to LIM: adenosine 3', 5'-cyclic monophosphate sodium salt monohydrate (cAMP) (Sigma #A6885) or 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR) (Sigma #A9978) were added to the cultures at various concentrations.

Results

Fet3p is a cell surface ferroxidase that regulates high-affinity iron uptake (Singh et al., 2006). Overexpression of *IZH2* causes repression of *FET3* activity; however, the signaling mechanism of how this occurs remains a mystery. We have already demonstrated that *IZH2*-dependent repression of *FET3* occurs through a competition between Nrg1p/Nrg2p and Msn2p/Msn4p. Thus, we examined the involvement of PKA and AMPK complexes, the upstream signaling proteins of both Nrg1p/Nrg2p and Msn2p/Msn4p, respectively.

PKA and Ras-cAMP is involved in *FET3* regulation

Previous studies have shown that PKA negatively regulates Msn2p/Msn4p activity and represses Aft1p-target genes (Robertson et al., 2000). PKA is made of three catalytic isoforms (Tpk1p, Tpk2p, and Tpk3p) and deletion of any of these isoforms results in loss of repression of p*FET3-lacZ* in iron-deficient LIM caused by *IZH2* (Figure 3-2A). The PKA complex is known to be positively regulated by the Ras2p-cAMP pathway (Jiang et al., 1998). This pathway involves Ras2p stimulating adenylate cyclase to produce cAMP, which, in turn activates PKA. Therefore we examined a strain missing *RAS2*, and found p*FET3-lacZ* repression by *IZH2* overexpression was also alleviated (Figure 3-2A). When cAMP was added to the *ras2Δ* mutant strain, *IZH2* overexpression was able to restore repression of p*FET3-lacZ* (Figure 3-2B).

AMPK is involved in *FET3* Regulation

AMP-dependent kinase (AMPK) is a negative regulator of Nrg1p and Nrg2p (Kuchin et al., 2002) and has been implicated in the regulation of iron-regulated genes (Haurie 2003 JBC). The AMPK complex is heterotrimer, which contains a catalytic α-subunit (Snf1p), a stimulatory γ-subunit (Snf4p), and one of three β-subunits (Gal83p, Sip1p, and Sip2p) that regulate AMPK cellular localization (Vincent et al., 2001). AMPK is activated by upstream kinases, AMPKK, of which there are three isoforms (Elm1p, Tos3p, and Sak1p) (Hedbacker et al., 2004).

Thus, we decided to examine if the AMPK subunits were involved in *IZH2*-dependent repression of p*FET3-lacZ*. We show that strains lacking Snf1p results in constitutive repression of ferroxidase activity (Figure 3-3A), while the loss of Gal83p or Sak1p, which are responsible for nuclear AMPK activity, also exhibits constitutive repression of p*FET3-lacZ* (Figure 3-3B). In addition, strains lacking the γ and cytoplasmic β subunits of AMPK (Snf4p, Sip1p, and Sip2p) resulted in a slight decrease in p*FET3-lacZ*. Moreover, in the *snf4Δ*, *sip1Δ*, and *sip2Δ* mutant strains, overexpression of *IZH2* was unable to repress p*FET3-lacZ* activity (Figure 3-3C). Furthermore, loss of Tos3p and Elm1p, two of the AMPKK isoforms, exhibited constitutive repression of p*FET3-lacZ* (Figure 3-3D). In this study, we also examined a known activator of mammalian AMPK, AICAR (Corton et al., 1995). When this molecule was added to our media, it produced a decrease in p*FET3-lacZ* activity (Figure 3-3E), suggesting that AMPK may play a role in signal transduction.

FET3* Regulation Requires *PKH1* and *PKH2

PKA has been shown to negatively regulate Msn2p/Msn4p. What, then, regulates PKA? One possibility is Pkh1p and Pkh2p. Pkh1p and Pkh2p are a pair of partially redundant kinases that are the yeast homologues of mammalian phosphoinositide-dependent kinase (PDK) (Casamayor et al, 1999). Deletion of Pkh1p and Pkh2p resulted in nearly complete loss of *IZH2*-

dependent repression of p*FET3-lacZ*, although Pkh1p seemed to have a greater role than Pkh2p (Figure 3-4A).

Mutant Strains do not Effect Aft1p Regulation

We were also interested to see if mutant strains affected the response of Aft1p to iron. Aft1p is a transcription factor that senses the availability of iron in the media (Dancis et al., 1992). Therefore, Aft1p should be turned on during iron deficiency and turned off during iron repletion. Our results demonstrate that deletion of *PKH1*, *PKH2*, *RAS2*, *TPK1*, *TPK2*, or *TPK3* exhibited induction of p*FET3-lacZ* (Figure 3-5A) in iron-defecient LIM. Also, yeast missing *SNF4*, *SIP1*, or *SIP2* (Figure 3-5B) demonstrated partial induction of p*FET3-lacZ*, whereas the loss of *GAL83*, *SAK1, TOS3, ELM1* (Figure 3-5B) or *SNF1* (Figure 3-5C) showed repression of p*FET3-lacZ* or ferroxidase activity in iron-defecient LIM.

Discussion

In this study, we showed that Izh2p affects the regulation of *FET3*. More importantly, we provide evidence that supports a new mechanism for *FET3* regulation. We intitially demonstrated that *IZH2* overexpression represses *FET3* by activating Nrg1p/Nrg2p and inactivating Msn2p/Msn4p. Thus, we proceeded to identify the upstream signaling proteins involved in regulation of NRG and MSN.

AMPK

One way in which *IZH2* could affect the competition between Nrg1p/Nrg2p and Msn2p/Msn4p is by activating Nrg1p/Nrg2p, as shown in chapter 2. The zinc finger transcriptional repressor proteins Nrg1p and Nrg2p are known to interact physically with and are negatively regulated by AMPK (Vyas et al., 2001). Therefore, it is possible that Izh2p activates Nrg1p/Nrg2p by inactivating AMPK.

The role of AMPK is complicated by its multiple functional isoforms that depend upon the cellular localization and regulatory subunits. Moreover, AMPK is activated by one of three AMPK kinases (Sak1p, Elm1p, and Tos3p) (Elbing et al, 2006). In yeast, most of AMPK's known functions occur in the nucleus, including Nrg1p/Nrg2p inactivation. We present evidence that nuclear AMPK is required for *FET3* transcription in low iron. This model is supported by the fact that strains lacking *SNF1*exhibited constitutive low *FET3* expression even in medium containing low iron. Furthermore, strains lacking *GAL83* and *SAK1*, which are responsible for active nuclear AMPK, exhibit the same constitutive *FET3* repression.

We designed experiments to test if cytoplasmic AMPK has a role in *IZH2*-dependent repression. Evidence for a role of cytoplasmic AMPK comes from data showing *FET3* repression by the addition of an AMPK activator (AICAR). Also, strains lacking the γ- and cytoplasmic β-subunits of AMPK (Snf4p, Sip1p, and Sip2p) are insensitive to *IZH2*-dependent repression. In addition, strains lacking any one of the three AMPK activating kinases exhibit constitutive *FET3* repression. These data support a model in which *IZH2* overexpression has opposite effects on nuclear and cytoplasmic isoforms of AMPK.

PKA's Role in *IZH2*-Dependent Repression.

Studies in yeast have shown that PKA represses Msn2p/Msn4p and regulates the growth and stress response (Smith et al., 1998). Furthermore, Izh2p has also been implicated in signaling through the Ras-cAMP/PKA pathway (Narasimhan et al., 2005). In addition, PKA is reported to regulate genes involved in iron homeostasis (Robertson et al., 2000). Finally, a report has also demonstrated that active PKA causes Snf1p to build-up in the cytoplasm (Hedbacker, et al., 2004) where it would have limited access to Nrg1p/Nrg2. This hypothesis is supported by our data which demonstrates the loss of any of the PKA catalytic subunits (Tpk1p, Tpk2p, and Tpk3p) eliminates *IZH2*-dependent repression of *FET3*.

We have also shown *FET3* repression via Izh2p requires Ras2p-cAMP signaling. However, is the Ras2p or production of cAMP required? We have demonstrated that the Ras2p is not essential, but the addition of cAMP was able to re-establish the ability of *IZH2* to repress *FET3* in a *ras2Δ* mutant strain. This data suggests that Izh2p is not directly coupled to Ras2p and is only required for the activation of PKA via cAMP.

Pkh1p/Pkh2p as a Master Regulator

If *IZH2*-dependent repression of *FET3* involves PKA, then what are the upstream proteins that transduce the signal? We suspected the involvement of a pair of partially redundant kinases known as Pkh1p and Pkh2p. This idea came from the fact that yeast PAQR homologues, *IZH1* and *IZH4*, are divergently transcribed from *PKH1* and *PKH2*. Divergently transcribed genes often belong in the same chemical pathway (Kruglyak and Tang, 2000). Also, Pkh1p and Pkh2p are homologues of the mammalian phosphoinositide-dependent protein kinase (PDK) (Casamayor et al, 1999). PDK is a central regulator that activates downstream kinases in the AGC superfamily (Vanhaesebroeck and Alessi, 2000). Thus, we looked at strains missing *PKH1* or *PKH2*, and found that *FET3* expression could not be repressed by *IZH2* overexpression. Even though Izh2p elicit a minimal effect in the *pkh1Δ* and *pkh2Δ* single mutant, we were unable to investigate the additive effect of losing both genes because the *pkh1Δpkh2Δ* double mutant strain is lethal (Inagaki et al.,1999). Furthermore, two-hybrid screens have demonstrated physical interactions between Pkh1p and the PKA isoform, Tpk3p (Ho et al., 2002).

Summation

Based on our data, we propose a model for signal transduction via *IZH2* overexpression that results in repression of high affinity iron uptake genes in yeast. Our data show that *IZH* overexpression activates Pkh1p/Pkh2p. Pkh1p/Pkh2p then regulates the activity of PKA and

AMPK and ultimately activates the transcription factors Nrg1p/Nrg2p and inactivates Msn2p/Msn4p.

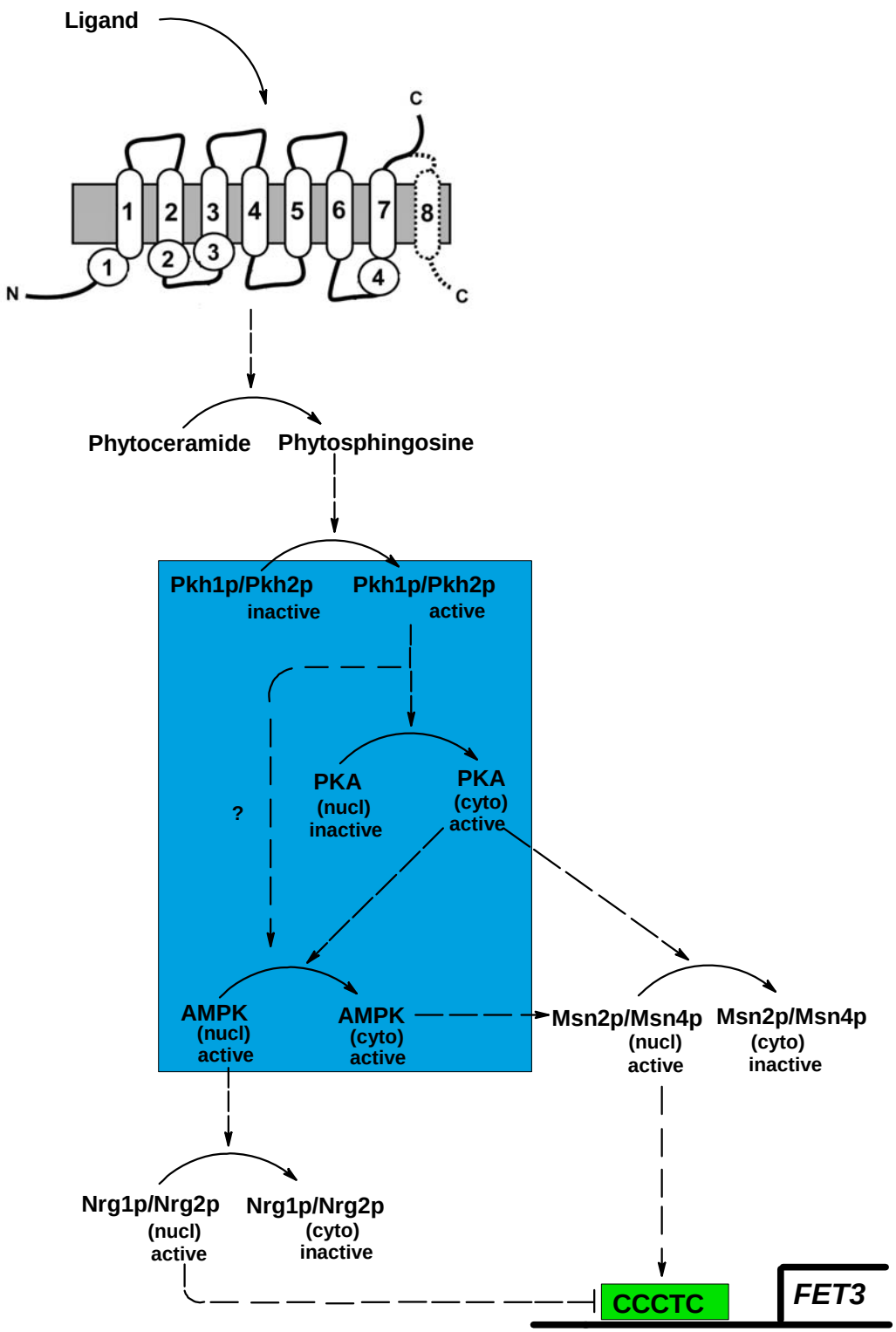


Figure 3-1. A model for the mechanism of *FET3* by *IZH2* overexpression. Solid arrows indicate protein activation or localization of protein. Broken arrows indicate protein interaction on localization and activation. Cyto = protein is cytoplamic. Nucl = protein is nuclear. Shaded blue portions represent areas studied in chapter.

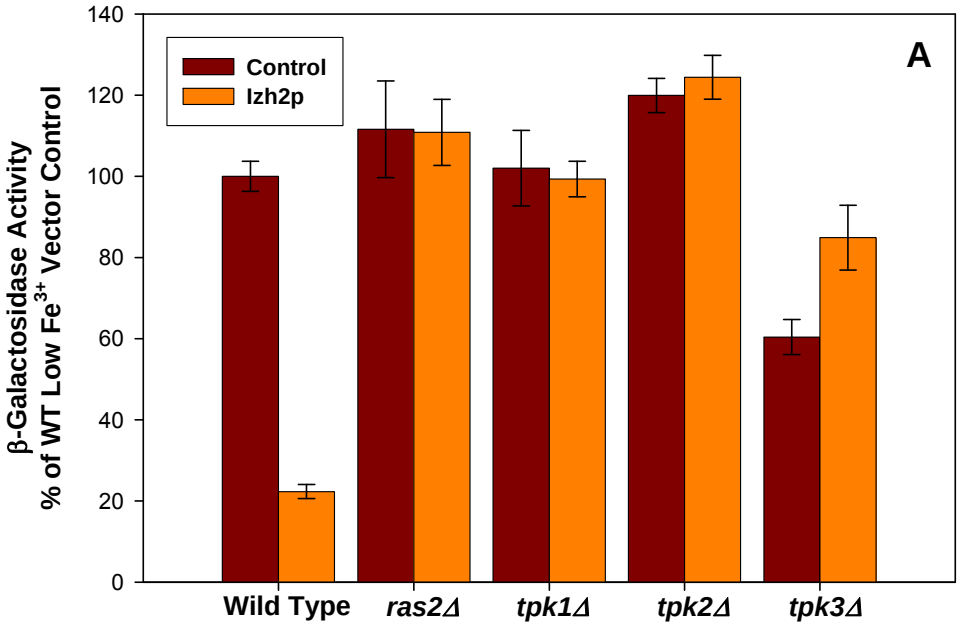


Figure 3-2. PKA and Ras-cAMP is involved in *FET3* regulation. BY4742 wild type and *ras2Δ* mutant strains are used in panels A and B. *tpk1Δ*, *tpk2Δ*, and *tpk3Δ* mutant strains are used in panel A. β-Galactosidase activities are shown as a percentage of fully induced activity in wild type strain carrying an empty control vector grown in iron-deficient LIM. The error bars represent ± 1 standard deviation for experiments performed in triplicate. β-Galactosidase activity of yeast strain carrying the p*FET3*-lacZ and the *IZH* overexpressors individually. (A) *IZH2* overexpression did not repress β-Galactosidase activity in *tpk1Δ*, *tpk2Δ*, *tpk3Δ*, and *ras2Δ* mutant strains. (B) *IZH2* overexpression in a *ras2Δ* mutant strain treated with 3 mM cAMP repressed p*FET3-lacZ* activity.

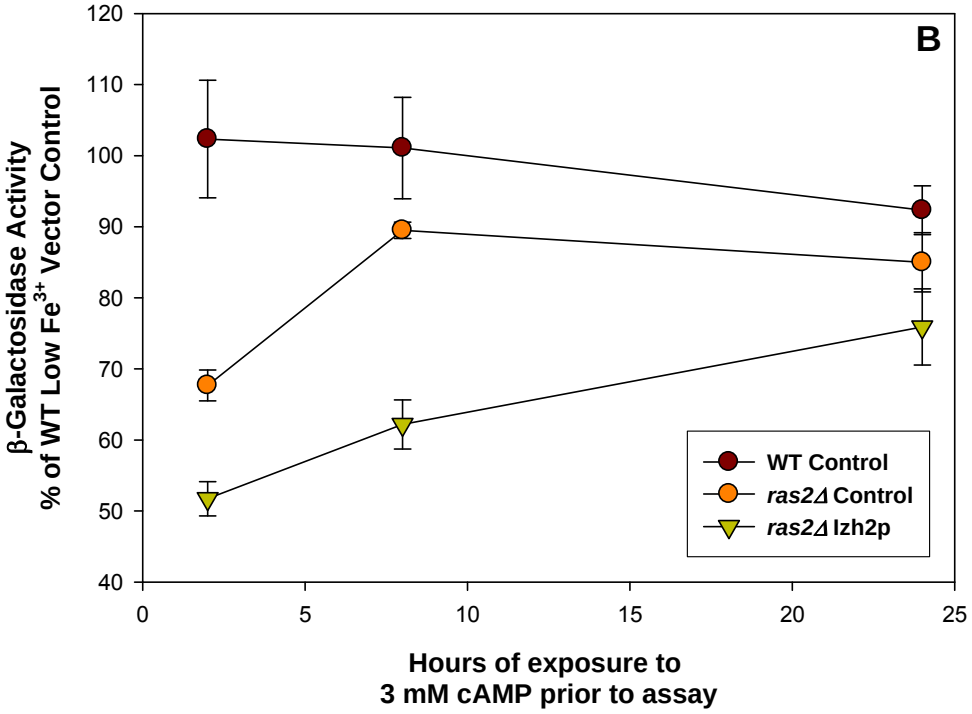


Figure 3-2 continued.

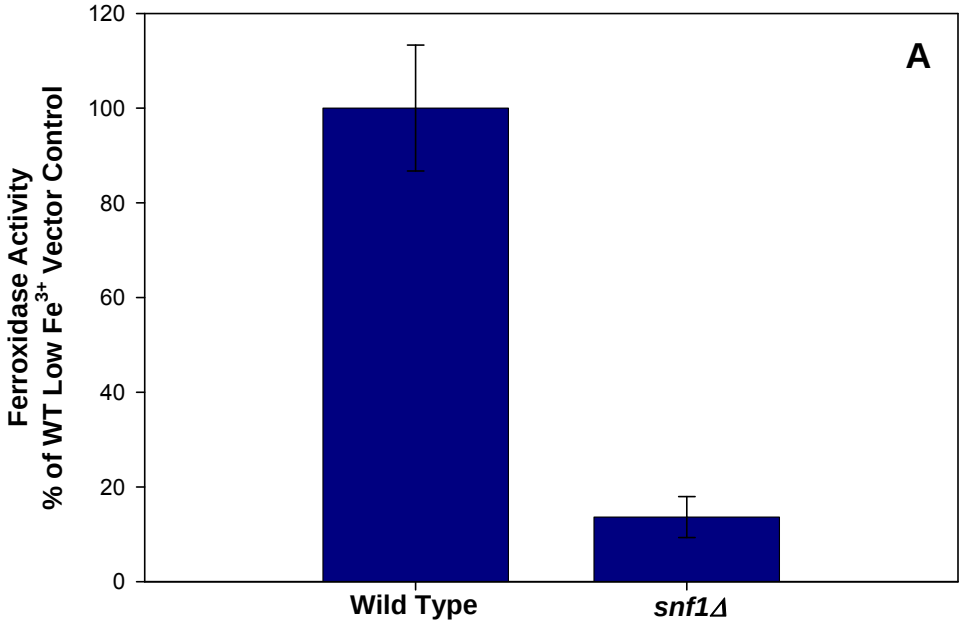
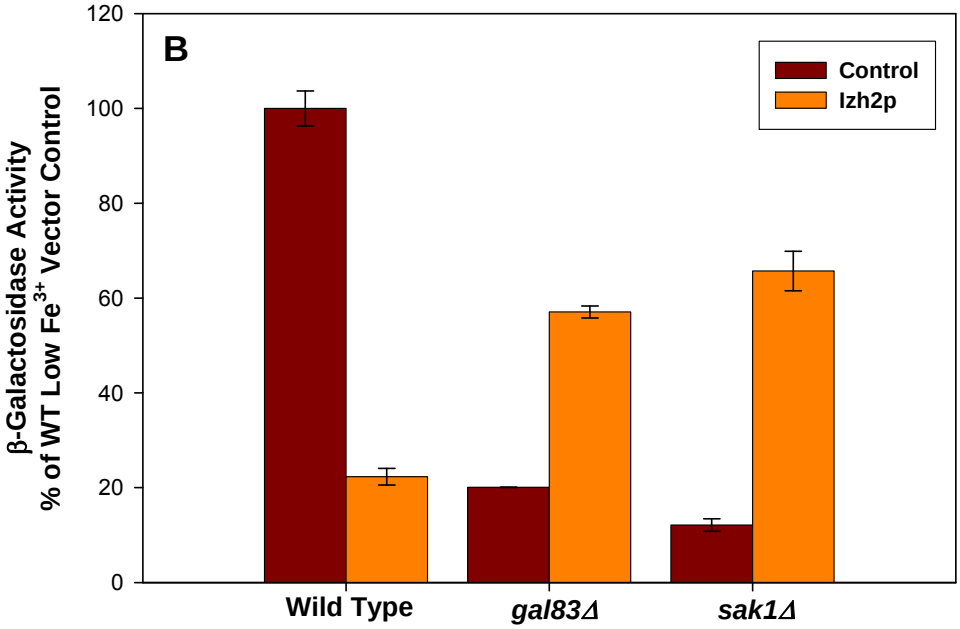


Figure 3-3. AMPK is involved in *FET3* regulation. BY4742 wild type strain was used in all panels. *snf1Δ*, *snf4Δ*, *gal83Δ*, *sip1Δ*, *sip2Δ*, *sak1Δ*, *tos3Δ*, and *elm1Δ* mutant strains were also used. β-Galactosidase (Panels B to E) or ferroxidase (Panel A) activities are shown as a percentage of fully induced activity in wild type strain carrying an empty control vector grown in iron-deficient LIM. The error bars represent ± 1 standard deviation for experiments performed in triplicate. (A) Ferroxidase activity is constitutively repressed in snf1Δ strain. (B) Deletion of *GAL83* and *SAK1* genes shows constitutive repression of p*FET3-lacZ*. (C) Deletion of *SNF4*, *SIP1*, and *SIP2* genes exhibit partial repression of p*FET3-lacZ*. *IZH2* overexpression had no affect in these strains. (D) Deletion of *TOS3* and *ELM1* genes showed constitutive repression of p*FET3-lacZ*. (E) Addition of 500 μM AICAR results in partial repression of p*FET3-lacZ*.



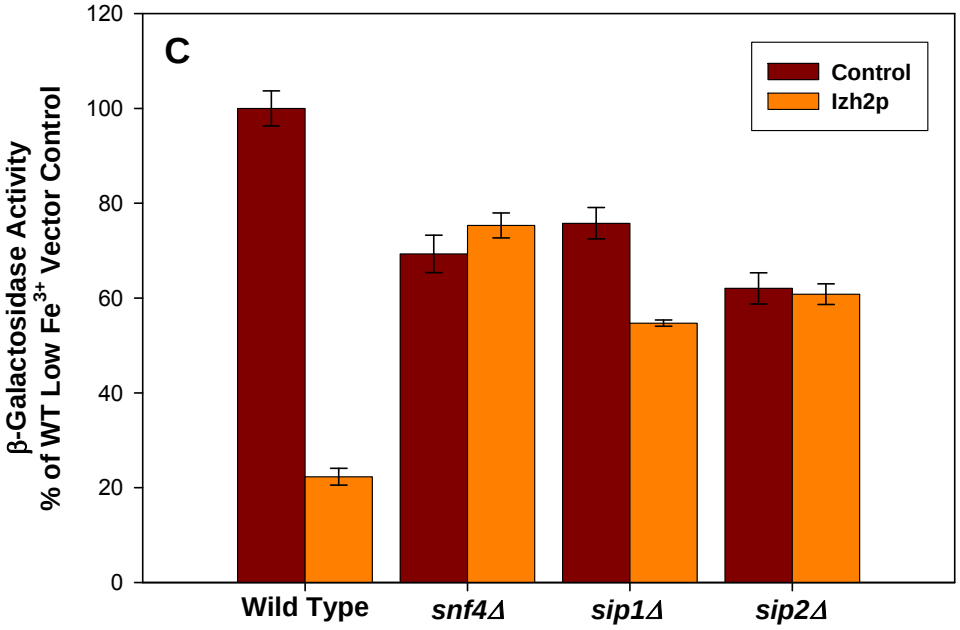
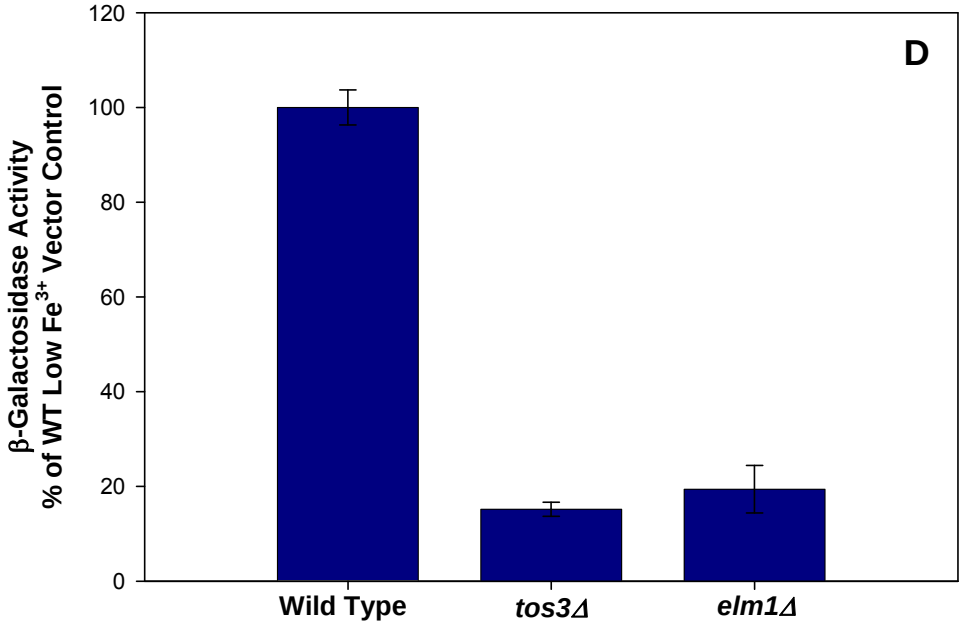


Figure 3-3 continued.



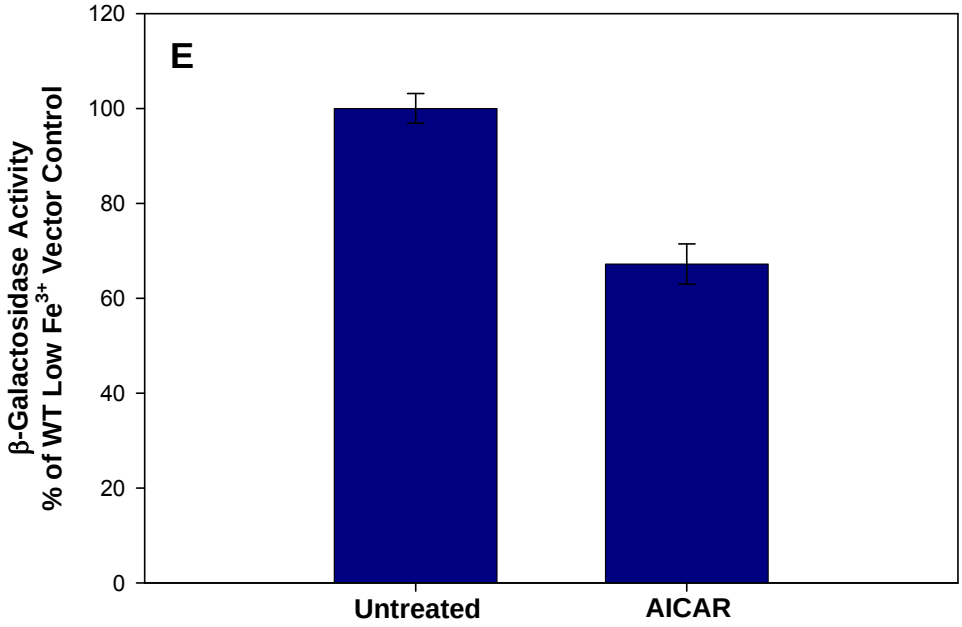


Figure 3-3 continued.

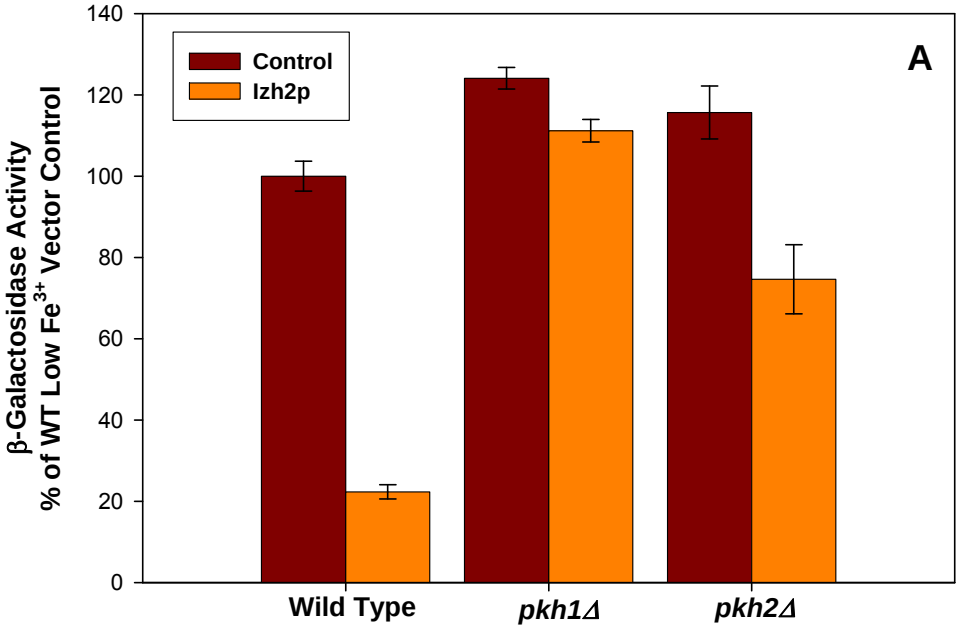


Figure 3-4. *FET3* regulation requires *PKH1* and *PKH2*. BY4742 wild type strain was used in panel A. *pkh1Δ* and *pkh2Δ* mutant strains are used in panel A. β-Galactosidase activities are shown as a percentage of fully induced activity in wild type strain carrying an empty control vector grown in iron-deficient LIM. The error bars represent ± 1 standard deviation for experiments performed in triplicate. β-Galactosidase activity of yeast strain carrying the p*FET3-lacZ* and the *IZH* overexpressors individually. (A) Deletion of *PKH1* and *PKH2* genes results an inability of the *IZH2* to repress p*FET3-lacZ*.

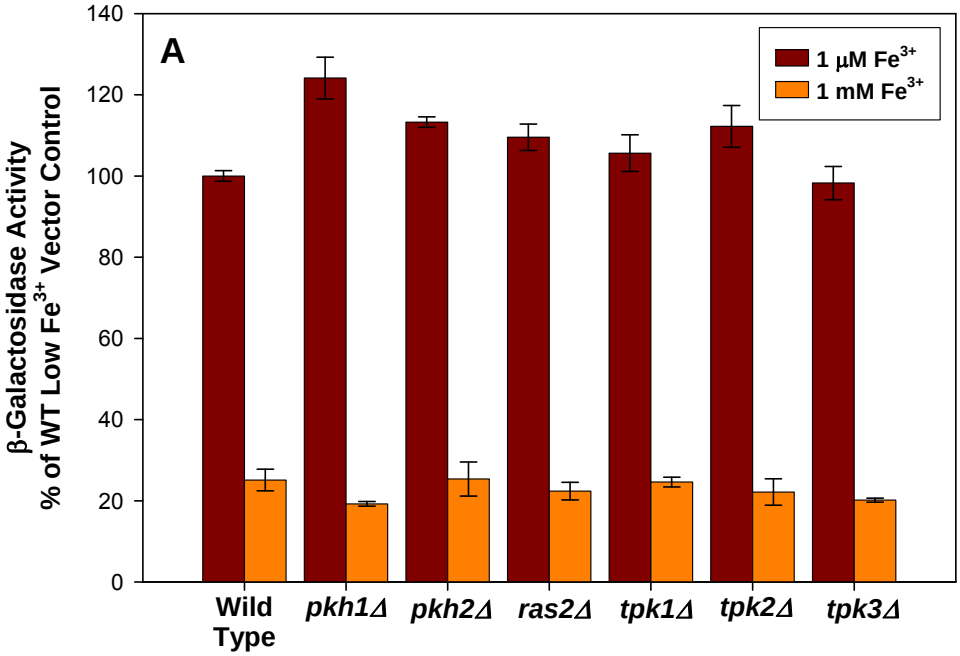
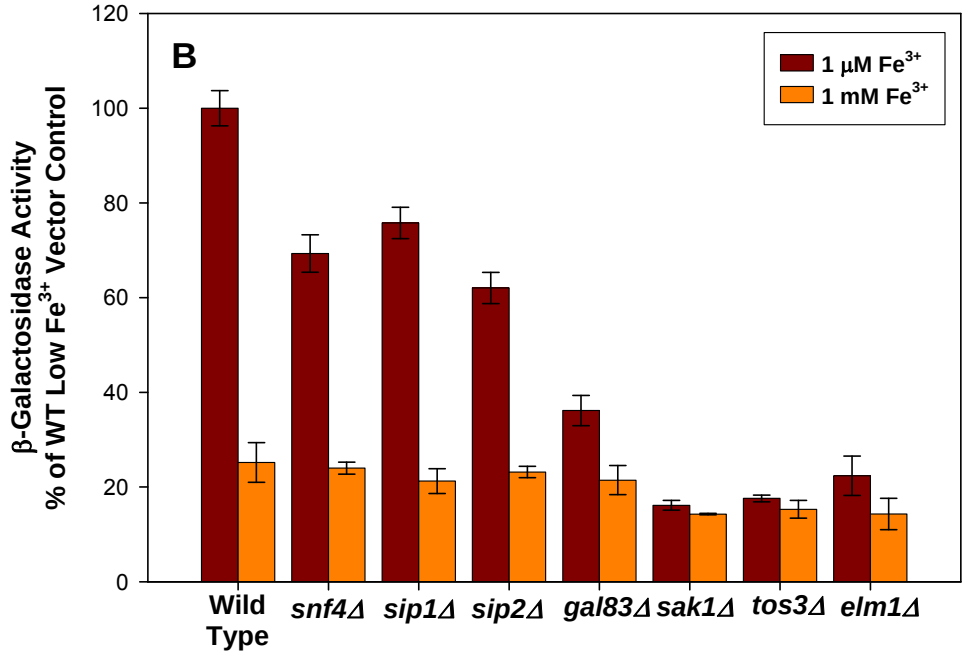


Figure 3-5. Aft1p responds normally to iron-defeciency in most mutant strains. BY4742 wild type strain was used in panels A, B, and C. β-Galactosidase (Panel A and B) or ferroxidase (Panel C) activities are shown as a percentage of fully induced activity in wild type strain carrying an empty control vector grown in iron-deficient LIM. The error bars represent ± 1 standard deviation for experiments performed in triplicate. (A) β-Galactosidase activities are induced in the BY4742 wild type stain, or strains lacking *PKH1*, *PKH2*, *RAS2*, *TPK1*, *TPK2*, or *TPK3*. (B) β-Galactosidase activities are partial induced in strains lacking *SNF4*, *SIP1*, or *SIP2*, but not in strains lacking *GAL83*, *SAK1, TOS3,* or *ELM1*. (C) Ferroxidase activity is induced in the BY4742 wild type stain, but not in strains lacking *SNF1*.



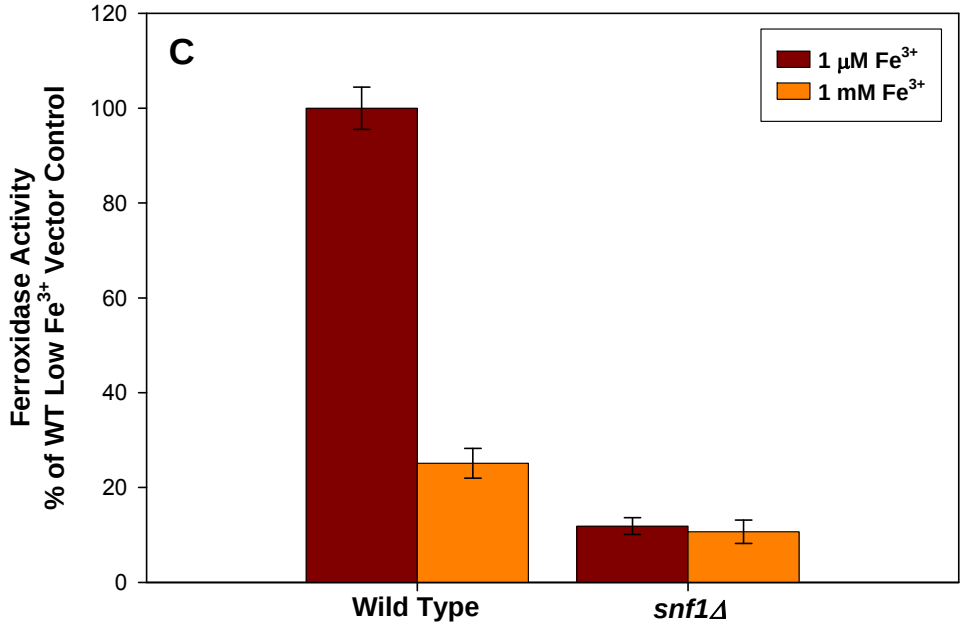


Figure 3-5 continued.

CHAPTER 4
PAQR-DEPENDENT REPRESSION OF *FET3* IS MEDIATED BY SPHINGOLIPIDS

Introduction

Sphingolipids, along with sterols, are important components of cell membranes in eukaryotic cells (Lisman et al., 2004). Their metabolites ceramide, sphingosine, and sphingosine-1-phosphate have important roles in cell biology (Merrill et al., 1989), particularly, their role in intracellular signaling. Sphingolipids, structurally, are amphipathic molecules that have hydrophobic and hydrophilic components. The hydrophobic region consists of a sphingoid long chain base (sphingosine in mammals or phytosphingosine in plants and fungi) to which a fatty acid is attached by an amide linkage to form a ceramide, which is the simplest sphingolipid. More complex sphingolipids have a polar head group consisting of a carbohydrate that is attached by glycosidic linkage to the ceramide at the C1-OH group are known as sphingoglycolipids.

Ceramides and sphingosine exert opposite effects in biological systems. Ceramide play a role in cellular growth, growth arrest, and stress responses (Merrill et al., 1997; Hannun and Obeid, 1997; Hannun, 1997; Hannun, 1996), while sphingosine is involved in stimulating growth and suppressing apoptosis (Cuvillier, 2002). Not surprisingly, the ratio of ceramide to sphingosine must be tightly regulated inside the cell. This ratio can be altered by enzymes. One such group of enzymes, ceramidases, plays an intricate role in maintaining a balance of this ratio. In *Saccharomyces cerevisiae*, the alkaline ceramidase, Ypc1p, deacylates phytoceramide to phytosphingosine and a free fatty acid. In addition, Ypc1p exhibits reverse activity forming phytoceramide from the free fatty acid and phytosphingosine (Hannun, 1996).

Ypc1p is structurally related to the PAQRs (Figure 4-1) (Lyons et al., 2004). Both the ceramides and PAQRs contain at least seven transmembrane domains (Figure 4-2) and a similar predicted topology with the cytoplasmic N-terminus and extra-cytoplasmic C-terminus. This predicted toplogy has been confirmed for AdipoR1, AdipoR2, Izh2p and Izh4p (Yamauchi et al., 2003; Kim et al., 2003; Kim et al, 2006). Since Ypc1p and the PAQR proteins are related, we investigated whether the Izh proteins also possess ceramidase activity.

In this study, we show that increasing endogenous sphingoid bases by overexpressing Ypc1p results in *FET3* repression by a mechanism that is similar to the *IZH*s. Furthermore, we show that adding exogenous sphingoid bases have the same effect on *FET3*. Finally we showed that the ceramidase inhibitor, D-erythro MAPP (Bielawska et al., 1996), alleviated *FET3* repression caused by *IZH* overexpression.

Thus, we propose that PAQR overexpression generates a sphingolipid second messenger (Figure 4-3) responsible for the downstream effects on *FET3*.

Materials and Methods

Yeast Strains and Plasmids

The W303-1A wild type strain and *ypc1Δydc1Δ* strain were a gift of Dr. Howard Rielzman (University Basel, Switzerland). Wild type (BY4742) strain and *pkh1Δ*, *pkh2Δ*, *tpk1Δ*, *tpk2Δ*, *tpk3Δ*, *ras2Δ*, *msn2Δ*, *msn4Δ*, *snf1Δ*, *snf4Δ*, *gal83Δ*, *sip1Δ*, *sip2Δ, nrg1, nrg2, lcb4Δ*, *lcb5Δ*, *sur1Δ*, *sur2Δ*, *sur4Δ*, *fen1Δ*, *scs7Δ*, *csg2Δ*, and *ipt1Δ* mutant strains were obtained from Euroscarf and their genotypes are listed in Supplementary Table 1. The p*FET3-lacZ* plasmids (Gift of D. Eide) has the promoter region of the *FET3* gene fused to the *lacZ* open reading frame and is *URA3* or *LEU2* selectable. *IZH2* was cloned from genomic DNA into pRS316-*GAL1* (*LEU2* or *URA3*), while YPC1 was cloned from genomic DNAinto pRS316-*GAL1* (*URA3*) via homologous recombination as previously described (Lyons et al., 2004). Primers used to clone

IZH2 and *YPC1* are listed in Supplementary Table 2. All clones were sequenced to ensure that no errors occurred in PCR.

β -Galactosidase and Ferroxidase Assays

β -Galactosidase and ferroxidase sssays were performed as described in chapter 2 except for the addition of the following compounds to LIM. Fresh stocks of sphingosine (SPH) (Avanti Polar Lipids #860490), phytosphingosine (PHS) (Avanti Polar Lipids #860499), dihydrosphingosine (DHS) (Avanti Polar Lipids #860498), and stearylamine (Sigma-Aldrich #124-30-1) were dissolved 1:1 in chloroform/methanol, fumonisin B_1 (FB_1) (Cayman #116355-83-0) in methanol, sphingosine kinase inhibitor 2 (SPH KI) (Cayman #312636-16-1) in dimethylsulfoxide, and N,N-dimethylsphingosine (N,N-DMS) (Cayman #119567-63-4), D-erythro-MAPP (Cayman #143492-38-0), and myriocin (Cayman #35891-70-4) in ethanol. For all experiments equal volumes of solvent were added to untreated cells to control for vehicle effects. For all cases, except myriocin, the drug was added upon reinoculation in LIM. In the case of myriocin, cells were treated with the drug for one hour prior to β-galactosidase or ferroxidase assay.

Results

Exogenous and Endogenous Sphingoid Bases Repress *FET3*

Since sphingoid bases are the only known activator of yeast Pkh1p and Pkh2p (Liu, et al., 2005), we decided to test their effect on *FET3* regulation. In this study, we show that the addition of 100 μM sphingosine, phytosphingosine, and dihydrosphingosine resulted in repression of p*FET3-lacZ*. However, another way to increase the concentration of sphingoid bases inside the cell is to overexpress Ypc1p, an alkaline ceramidase that catalyzes the hydrolysis of phytoceramide to phytosphingosine (Mao et al., 2000). Overexpression of *YPC1* also resulted in repression of p*FET3-lacZ* activity.

Exogenous and Endogenous Sphingoid Bases Mimic the Effect of *IZH2* Overexpression

Both overexpression of Ypc1p and addition of phytosphingosine or dihydrosphingosine (Figures 4-4A and 4-4B) caused p*FET-lacZ* or ferroxidase repression that depends on *PKH1*, *PKH2*, *TPK1*, *TPK2*, *TPK3*, *RAS2*, *SNF4*, *SIP1*, S*IP2*, *NRG1*, and *NRG2*. Deletion of *PKH1*, *PKH2*, *TPK1*, *TPK2*, *TPK3*, *NRG1*, and *NRG2* resulted in alleviation of p*FET3-lacZ* repression caused by a break in the signal transduction pathway (Figures 4-5A, 4-5E, and 4-5F), while only partial alleviation was demonstrated in the *snf4Δ*, *sip1Δ*, and *sip2Δ* mutant strains (Figure 4-5D). This data suggests that Ypc1p, phytosphingosine, or dihydrosphingosine activates a similar pathway as PAQR overexpression (Kupchak et al., 2007).

***IZH2* Overexpression is Affecting Sphingoid Metabolism**

In this study, we postulated that *IZH2* may require the *de novo* biosynthesis of sphingoid bases and ceramidases by affecting serine palmitoyltransferase (SPT). To test this hypothesis, we used a SPT inhibitor, myriocin (Miyake et al., 1995) (Figure 6-6A). We showed that myriocin fully alleviated p*FET3-lacZ* repression of *IZH2* overexpression and demonstrated maximal effect at 10 μM (Figure 4-7A). But, myriocin did not alleviate p*FET3-lacZ* repression by *YPC1* overexpression, phytosphingosine addition, or in iron-replete LIM (Figure 4-7B).

Sphingoid bases can also accumulate by inhibiting ceramide synthases Lag1p and Lac1p, via the antifungal drug fumonisin B_1 (FB_1) (Wang et al., 1991). Thus, we examined the effect of fumonisin B_1 on p*FET3-lacZ* activity (Figure 4-6B). p*FET3-lacZ* is partially repressed at 1 μM FB_1 and maximum repression occurs at 10 μM FB_1 (Figure 4-6C). We also tested the ability of two inhibitors of sphingosine kinase (SK), N,N-dimetylsphingosine (N,N-DMS) and sphingosine kinase inhibitor (SPH KI) to block the conversion of sphingosine to sphingosine-1-phosphate (Figure 4-6C). p*FET3-lacZ* is fully repressed by the addition of 10 nM N,N-DMS, while SPH

KI repressed p*FET3-lacZ* at 10 μM in the BY4742 wild strain carrying the empty vector grown in iron-defecient LIM (Figure 4-7C).

Accumulation of Sphingoid Bases Effects *FET3*

To further examine sphingoid base accumulation, we investigated the effects of deleting genes involved in complex sphingolipid formation. Therefore, we looked at p*FET3-lacZ* activity in strains lacking *SCS7*, *SUR1*, *CSG2*, *IPT1*. We show that *scs7Δ*, *sur1Δ*, *csg2Δ*, and *ipt1Δ* mutant strains exhibited constitutive repression of p*FET3-lacZ* in iron-deficient LIM (Figure 4-8A). In this study, we also investigated the effect of deleting *SUR2*, which is responsible for converting dihydrosphingosine into phytosphingosine (Haak et al., 1997). In the *sur2Δ* mutant strain p*FET3-lacZ* is constitutively repressed (Figure 4-8B). Also, we studied strains lacking, either, long chain fatty acid elongases, *sur4Δ* and *fen1Δ*, or long chain base kinases, *lcb4Δ* and *lcb5Δ*. These strains all exhibited constitutive repression of p*FET3-lacZ* activity; however, *IZH2* overexpression did not affect p*FET3-lacZ* in these strains in iron-deficient LIM (Figures 4-8C).

Endogenous Alkaline Ceramidase are not Required for *FET3* Signaling

In this study, we also investigated *IZH2* ability to act as an alkaline ceramidase. We measured the effect of D-erythro-MAPP (Bielawska et al., 1996) (Figure 4-9), an inhibitor of *YPC1* and *YDC1*, on Izh2p-dependent repression p*FET3-lacZ* repression. D-erythro-MAPP alleviated repression of p*FET3-lacZ* by *IZH2* overexpression and exhibits its maximal effect at 100 nM in iron-deficient LIM. It also eliminated the repression caused by *YPC1* overexpression on p*FET3-lacZ*; however, D-erythro-MAPP did not have an effect on p*FET3-lacZ* repression when phytosphingosine was added to iron-deficient LIM or when the experiment was performed in iron-replete LIM (Figure 4-10A).

We also studied the effect of *IZH2* overexpression in a strain lacking *YPC1* and *YDC1*, which are the endogenous alkaline ceramidases. Unexpectedly, ferroxidase activity was partially

repressed in this strain. Nevertheless, Izh2p overexpression slightly repressed ferroxidase activity, while Ypc1p overexpression fully repressed ferroxidase activity in iron-defecient LIM (Figure 4-10B). We also examined the accumulation of sphingoid bases by inhibiting serine palmitoyltransferase, ceramide synthase, and sphingosine kinase in the *ypc1Δydc1Δ* mutant strain. Ferroxidase activity in the *ypc1Δydc1Δ* strain was partially alleviated by the addition of 100 nM D-*erythro*-MAPP and 1 μM myriocin, but not by 10 nM N,N-DMS in iron-deficient LIM (Figure 4-10C).

Discussion

The Izh proteins in yeast belong to a large family of membrane receptors known as PAQRs, which also include related proteins from human and bacteria. The PAQR family is unified by the presence of seven transmembrane domains and three highly conserved, short, amino acid motifs (Lyons et al., 2004). The PAQRs also share distant similarity with a family of proteins known as the alkaline ceramidases as shown in chapter 1. Therefore, the similarities between the PAQRs and the alkaline ceramidases suggest they both may possess the same enzymatic activity.

Effect of Sphingoid Bases on *FET3*

Similar to the Izh2p, the overexpression of *YPC1*, an alkaline ceramidase that hydrolyzes phytoceramide to phytosphingosine, repressed *FET3*. This effect on *FET3* could also be recapitulated by the addition of exogenous sphingoid bases. In this case, sphingosine (SPH), phytosphingosine (PHS), and dihydrosphingosine (DHS) all repressed *FET3*. Also molecules that had a similar structure to the sphingoid bases, such as stearylamine, could not repress *FET3* suggesting that the effect is specific for sphingoid bases.

Even though the addition of exogenous sphingoid bases can cause an effect on *FET3*, this does not mean that sphingoid bases and the Izh2p represses *FET3* by identical mechanisms. In

yeast, sphingoid bases are known to activate Pkh1p and Pkh2p. This is consistent with our hypothesis that sphingoid bases act as a second messenger in the repression of *FET3*. As with *IZH* overexpression, *FET3* repression by exogenous sphingoid bases or by *YPC1* overexpression also requires Pkh1p/Pkh2p, PKA, AMPK, and Nrg1p/Nrg2p. Thus suggesting, with minor exceptions, that Izh2p and sphingoid bases may activate a similar pathway in yeast.

Izh2p-Dependent Signaling is Alleviated By Disruption of Sphingoid Base Synthesis

The question arose of how Izh2p affects sphingoid base levels. The Izh2p could alter sphingoid base levels in two ways: 1) it could increase *de novo* biosynthesis by affecting serine palmityltransferase (SPT) or, 2) increase catabolism of ceramide by activating ceramidase activity. Therefore, we first tested *IZH2* overexpression effect on *de novo* sphingoid base biosynthesis. *De novo* sphingoid base biosynthesis begins in the endoplasmic reticulum where SPT, which is composed of Lcb1p, Lcb2 and Tsc3p subunits, catalyzes the condensation of serine with fatty acid-CoA to yield 3-ketosphinganine (Hanada et al., 2003). *Tsc10* catalyzes the reduction of 3-ketosphinganine to dihydrosphingosine (Beeler et al., 1998), which is then hydroxylated by *SUR2* to form PHS (Haak et al., 1997).

To test if *IZH2* overexpression effects *de novo* sphingoid base biosynthesis, we added the SPT inhibitor, myriocin. Treatment with myriocin caused alleviation of Izh2p-dependent repression of *FET3* in a dose dependent manner. However, the addition of myriocin could not effect *FET3* repression when YPC1 was overexpressed, or when we added PHS or iron to the media. This suggests myriocin does not cause a general effect on *FET3* transcription.

Accumulation of Sphingoid Bases Represses *FET3*

The fact that myriocin treatment alleviates Izh2p-dependent repression of *FET3*, indicates signaling must require *de novo* sphingoid base biosynthesis. We therefore tested if *de novo* ceramide biosynthesis was also required. The ceramide synthases, Lag1p and Lac1p, catalyze

the formation of ceramidases from sphingoid bases (Guillas et al., 2001; Schorling et al., 2001). Lag1p and Lac1p are inhibited by fumonisin B_1 (FB_1), which caused the build-up of sphingoid bases. The addition of fumonisin to cells carrying the empty vector exhibited constitutive repression of *FET3*. Thus *IZH2*-dependent repression of *FET3* is inhibited by myriocin, while FB_1 constitutively activates it. This result suggests that sphingoid base accumulation may function downstream of Izh2p in this signal transduction pathway.

Accumulaton of sphingoid bases can also be generated in a variety of other artificial conditions, and could be accomplished through inhibiting sphingoid base degradation. Excess sphingoid base is degraded by a pair of long chain base (LCB) kinases, Lcb4p and Lcb5p, which phosphorylate the sphingoid base to make sphingoid base-1-phosphate. In *S. cerevisiae*, Lcb4p accounts for the majority of LCB kinase activity (Nagiec et al., 1998). The newly formed phytosphingosine-1-phosphate (PHS-1-P) or dihydrosphingosine-1-phosphate DHS-1-P can then be cleaved by an LCB phosphate lyase, Dpl1p (Saba et al., 1997), to yield ethanolamine phosphate and a C16 aldehyde. We postulated that since sphingoid bases accumulate in strains lacking Lcb4p or Lcb5p, or when LCB kinases are inhibited that a decrease in sphingoid base phosphorylation would result in repression of *FET3*. First, we tested the effects of the LCB kinase inhibitors, N,N-dimethylsphingosine (N,N-DMS) and sphingosine kinase inhibitor 2 (SPH KI), and found that both N,N-DMS and SPH KI repressed *FET3* in an Izh2p-independent manner. Second, in cells lacking *LCB4* or *LCB5*, *FET3* is also constitutively repressed. However, the loss of *LCB4* has a more significant effect on *FET3* than the loss of *LCB5*, suggesting *LCB4* consistent with its role in sphingoid base phosphorylation.

A second way to create the accumulation of PHS is to delete the genes involved in the synthesis of complex sphingolipids. Once a ceramide is formed, it is transferred to the golgi

apparatus, where it is hydroxylates the ceramide fatty acid by Scs7p (Dunn et al., 1998). Inositol phosphate is then placed onto the ceramide by Aur1p to form inositol phosphoceramide (IPC) (Haak et al., 1997). The enzyme complex Sur1p, Csg2p, and Csh1p then catalyzes the formation of mannose-inositol-phosphoceramide (MIPC) from GDP-mannose (Beeler et al., 1997) (Uemura et al., 2003) (Lisman et al., 2004). The formation of the most complex sphingolipid in yeast, mannose-(inositol-phosphate)$_2$-ceramide (M(IP)$_2$C), is made by the transfer of a second inositol phosphate to MIPC, a reaction that requires Ipt1p (Dickson, et al., 1997). Therefore, we decided to test strains that were lacking these genes involved in complex sphingolipid biosynthesis except for the *aur1Δ* mutant strain, which is lethal (Nagiec et al., 1997). In the *scs7Δ*, *sur1Δ*, *csg2Δ*, or *ipt1Δ* mutant strains, *FET3* was constitutively repressed suggesting a reversal of this pathway and a build-up of sphingoid bases.

Another way to create excess amounts of PHS is to delete *SUR4* or *FEN1* genes. Both of these genes are essential for the conversion of C16 fatty acids to very long chain fatty acids (C20 to C26) that are required for ceramides (Oh et al., 1997). Therefore, we tested the effect of knocking out *SUR4* and *FEN1* on *FET3*. In *sur4Δ* and fen*1Δ* mutant strains, *FET3* exhibited constitutive repression.

We were also interested if the accumulation of excess endogenous DHS would have the same effect as the addition of exogenous DHS. Accumulation of DHS can occur by the deletion of the *SUR2* gene, which is responsible for converting DHS into PHS (Haak et al., 1997). In the *sur2Δ* mutant strain, *FET3* is constitutively repressed suggesting that PHS and DHS have a similar effect on *FET3*.

Inhibition of Ceramidase Activity Alleviate *IZH*s and *YPC1* Effect on *FET3*

The Izh2p could also alter sphingoid base levels by increasing catabolism of ceramide by activating ceramidase activity. First, we measured the effect of D-*erythro*-MAPP, an alkaline

ceramidase inhibitor, on *IZH2*-dependent repression of *FET3* and found that D-*erythro*-MAPP was able to partially alleviate the repression of *FET3* by both *YPC1* and *IZH2* overexpression.

To verify our results that *IZH* overexpression was causing the accumulation of sphingoid bases, Nancy Villa studied accumulation of sphingoid bases by overexpressing Izh2p. In the experiment, she found a modest accumulation of both phytosphingosine and dihydrosphingosine compared to vector control. Moreover, this accumulation suggests that the Izh2p may be acting as ceramidases and that Izh2- and Ypc1p-dependent signaling requires the conversion of sphingoid bases to ceramides prior to the formation of the sphingoid second messenger responsible for *FET3* repression.

***FET3* Signaling does not Require Endogenous Alkaline Ceramidases**

Previous results have shown myriocin inhibits Izh2p-dependendent *FET3* repression, but not Ypcp1p- or exogenous PHS-dependent repression of *FET3* in a wild type strain. This suggests Izh2p requires newly synthesized ceramides for its effect on *FET3,* while Ypc1p does not. This was our first indication that Izh2p does not work through Ypc1p. In order to study Ypc1p role in Izh2p signaling, we obtained a strain lacking *YPC1* and *YDC1*. This strain was used because it has no endogenous ceramidase activity. Therefore, we tested if Izh2p could repress *FET3* in *ypc1Δydc1Δ* mutant strain. Strikingly, *FET3* was partially repressed in this strain; however, Izh2p overexpression did slightly enhance *FET3* repression. This suggests Izh2p-dependent signaling is independent of alkaline ceramidases. The low expression of *FET3* in the *ypc1Δydc1Δ* mutant strain can be explained by a decrease in ceramide turnover resulting in an increase of PHS/DHS accumulation by feedback inhibition. This result is supported by myriocin alleviating the constitutive repression of *FET3*, while N,N-DMS further represses *FET3* (Figure 4-11).

Summation

The PAQR family shares similarities with the alkaline ceramidases. This led us to postulate that the Izh proteins may be acting as ligand-regulated ceramidases that control the balance between ceramides and sphingoid bases. Our data supports two main conclusions. First, repression of *FET3* by *IZH*-dependent signaling requires *de novo* ceramide biosynthesis and that this signal transduction on *FET3* can be stopped by inhibition of ceramidase activity. Second, sphingoid bases acts a second messenger and signals *FET3* repression via a similar mechanism as seen by the yeast and human PAQRs.

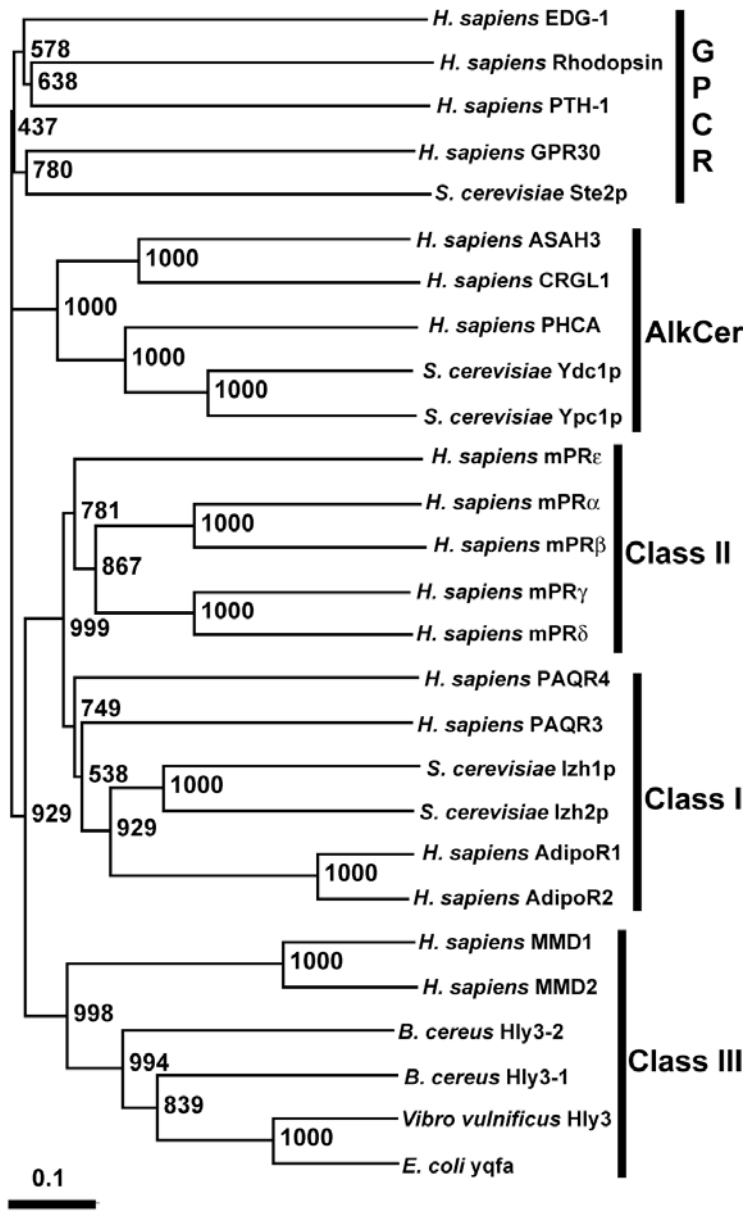


Figure 4-1. Phylogenetic analysis of PAQRs. A bootstrapped phylogenetic tree is shown the relationship between the PAQRs from various sources. Izh1p and Izh2p cluster with AdipoR1 and AdipoR2. Yeast and human GPCRs form a distinct clade and are included as an outgroup, which the tree is rooted. The length of the tree branches is proportional to the calculated distance between sequences with the scale bar indicating 0.1 substitutions per site. Numbers at the nodes are confidence values that refer to the number of times per 1,000 trees drawn a particular grouping is made.

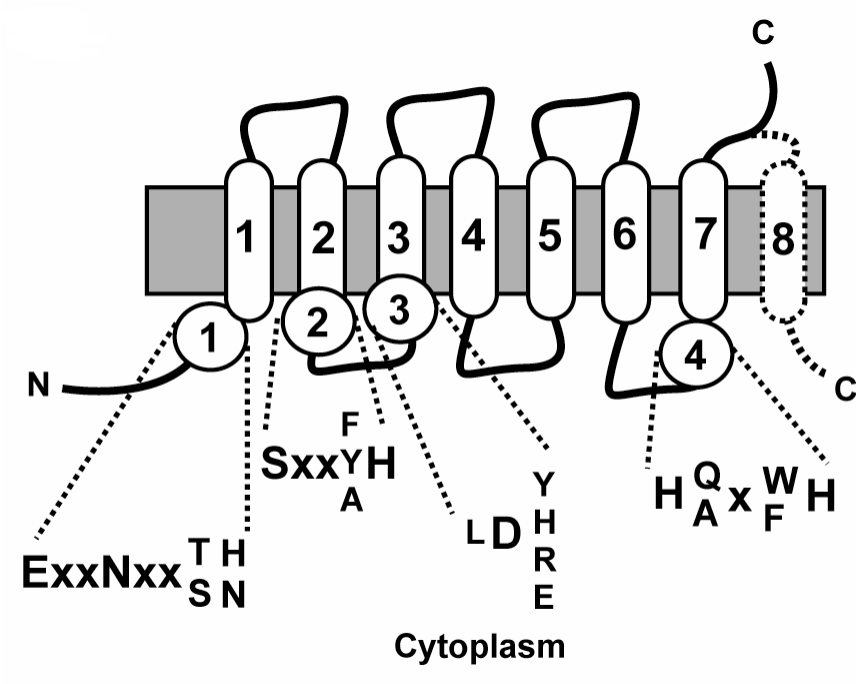


Figure 4-2. Topology predicted for all members of the PAQRs and alkaline ceramidases with the locations and the consensus sequences of the three highly conserved motifs. Grey represents membrane, ovals indicate transmebrane domains, and circles represent conserved regions of PAQR family. Class I, III PAQRs, and alkaline ceramidases are presumed to have 7 transmembrane domains while Class II PAQRs may have 8 transmembrane domains.

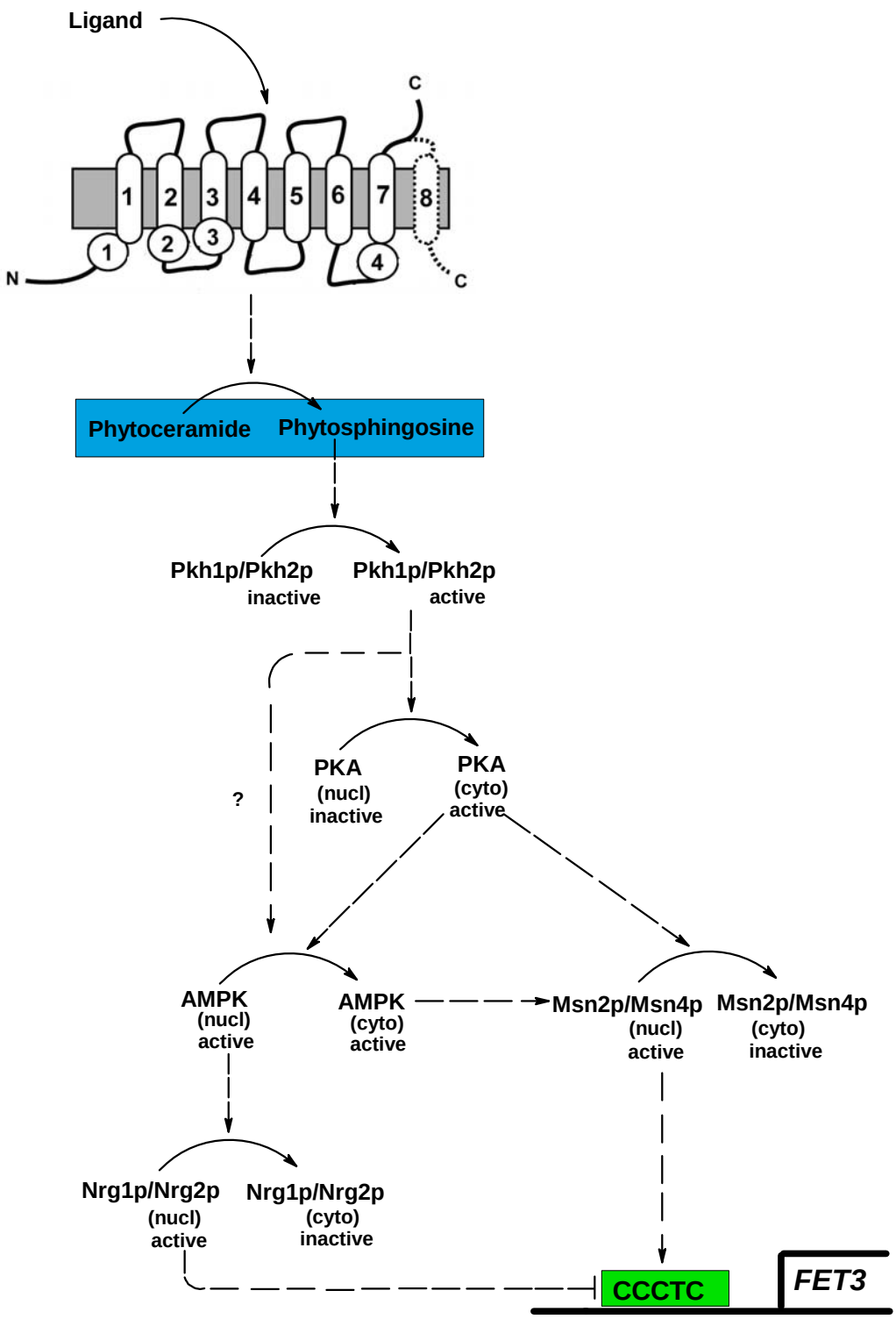


Figure 4-3. A model for the mechanism of *FET3* by *IZH2* overexpression. Solid arrows indicate protein activation or localization of protein. Broken arrows indicate protein interaction on localization and activation. Cyto = protein is cytoplamic. Nucl = protein is nuclear. Shaded blue portions represent areas studied in chapter.

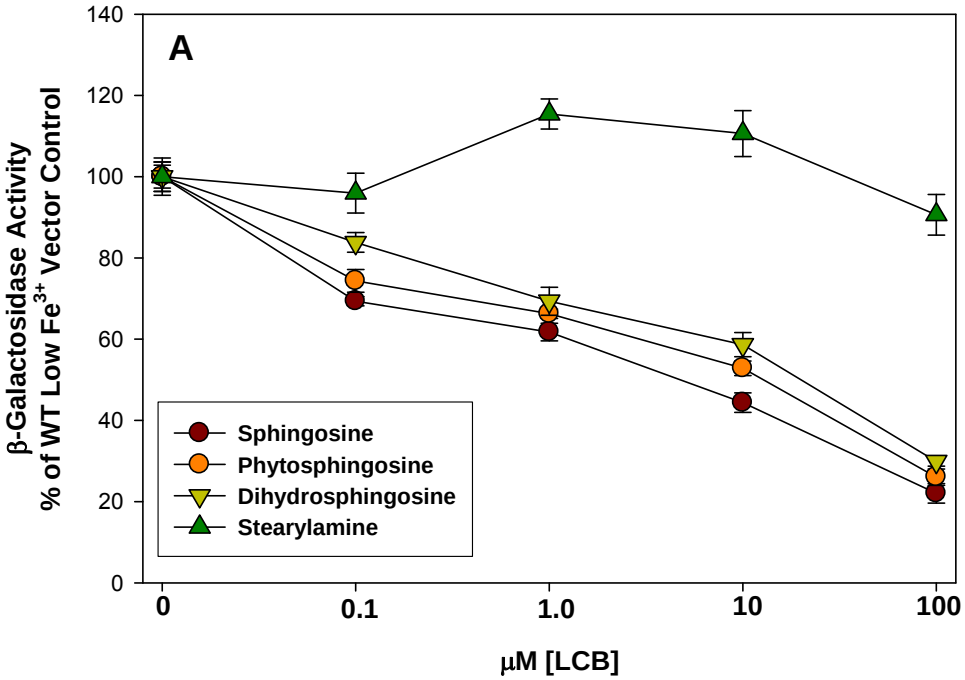
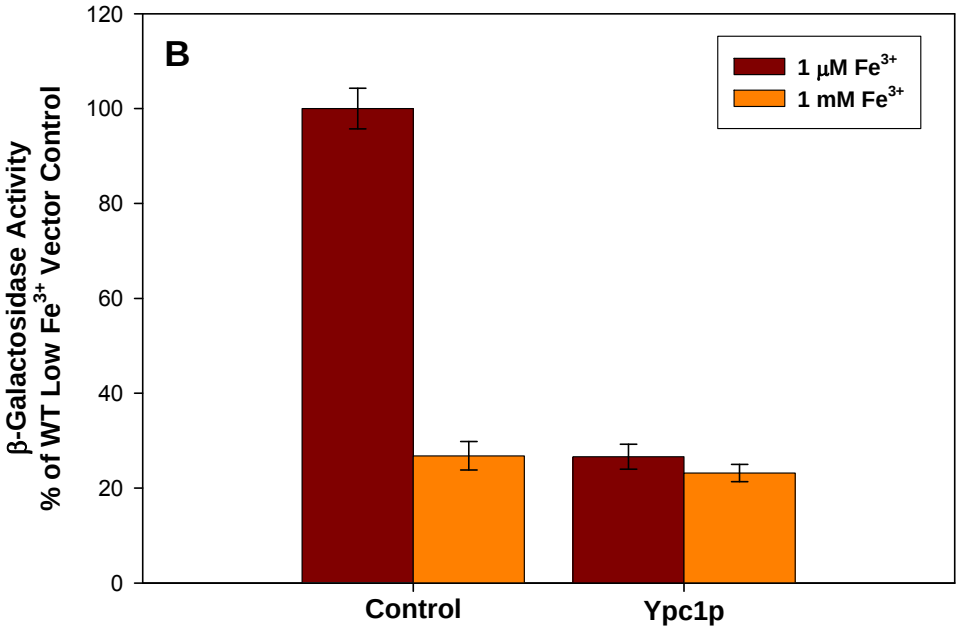


Figure 4-4. Exogenous and endogenous sphingoid bases affect *FET3*. BY4742 wild type strain is used in panels A and B. β-Galactosidase activities are shown as a percentage of fully induced β-galactosidase in wild type strain carrying an empty control vector grown in iron-deficient or iron-replete LIM. The error bars represent ± 1 standard deviation for experiments performed in triplicate. (A) β-Galactosidase activities of the p*FET3-lacZ* reporter are induced in iron-deficient LIM in the wild type strain when sphingosine, phytosphingosine, or dihydrosphingosine was added, but not with stearylamine. (B) *YPC1* overexpression exhibited an effect on the p*FET3-lacZ* reporter in iron-deficient LIM.



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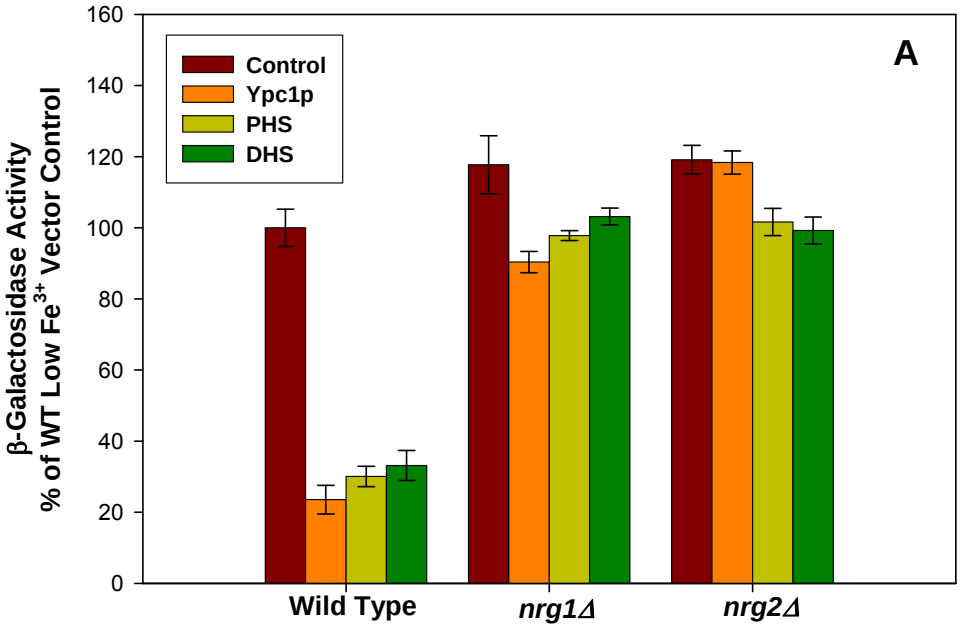
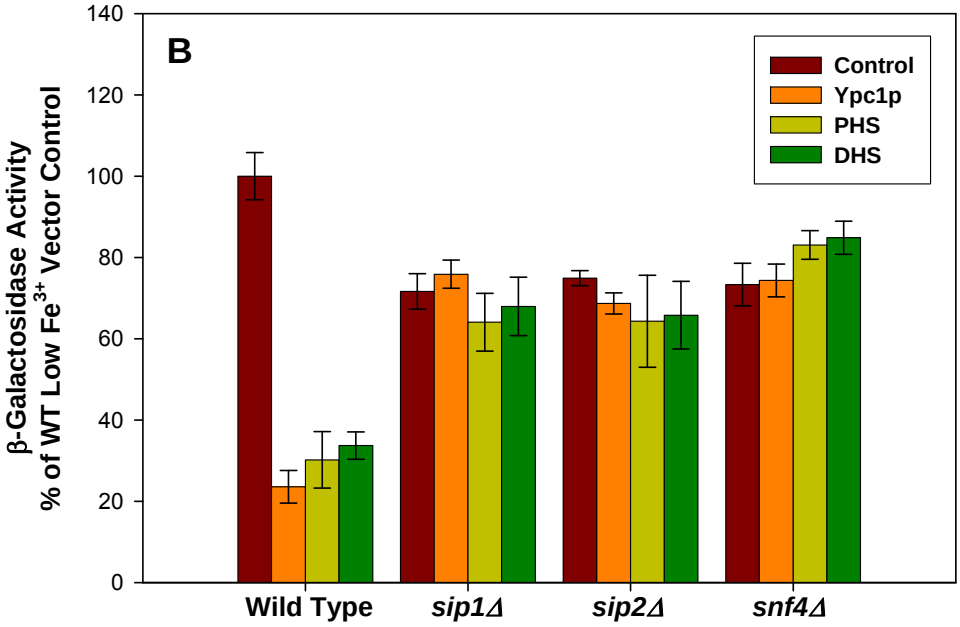
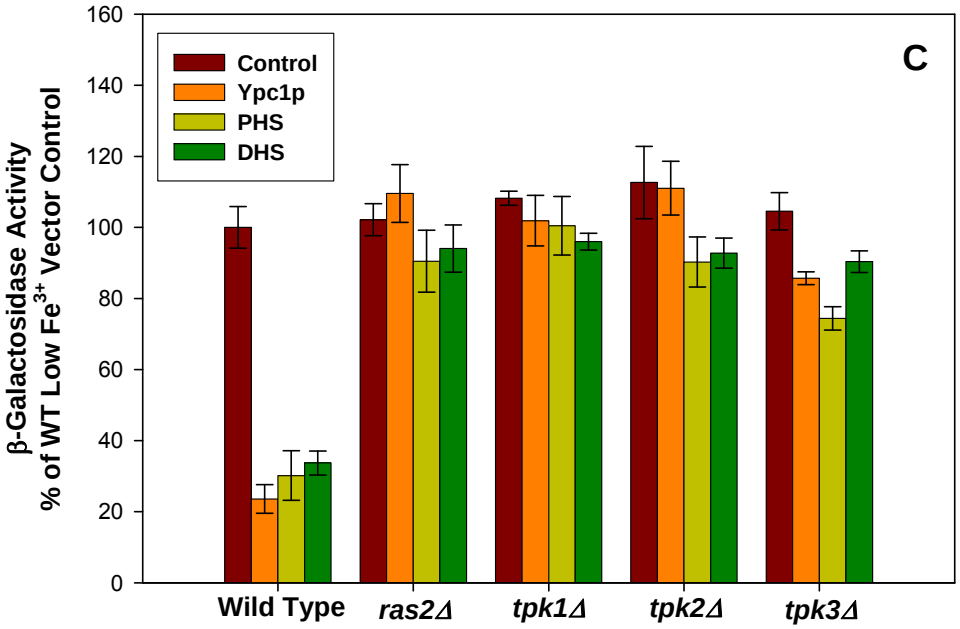


Figure 4-5. Sphingoid bases cause an *IZH*-dependent repression of *FET3*. BY4742 wild type strain is used in panels A to D. β-Galactosidase activities are shown as a percentage of fully induced β-galactosidase activity in wild type strain carrying an empty control vector grown in iron-deficient or iron-replete LIM. The error bars represent ± 1 standard deviation for experiments performed in triplicate. (A) Loss of *NRG1* and *NRG2* exhibit loss of repression of p*FET3-lacZ*. (B) β-Galactosidase activities of the p*Fet3-lacZ* reporter are repressed in strains lacking *GAL83*. Ypc1p overexpression partially alleviated *pFET3-lacZ* repression. Strains lacking AMPK β (Snf4p) or γ (Sip1p and Sip2p)-subunits partially alleviated p*FET3-lacZ* repression when Ypc1p was overexpressed or with the addition of phytosphingosine or dihydrosphingosine. (C) β-Galactosidase activities of the p*Fet3-lacZ* reporter are alleviated in strains lacking *TPK1*, *TPK2*, and *TPK3*, but only partially alleviated in the *RAS2* deleted strain when Ypc1p was overexpressed or phytosphingosine or dihydrosphingosine was added to iron-deficient LIM. (D) β-Galactosidase activities of the p*FET3-lacZ* reporter are alleviated in strains lacking *PKH1* and *PKH2*.





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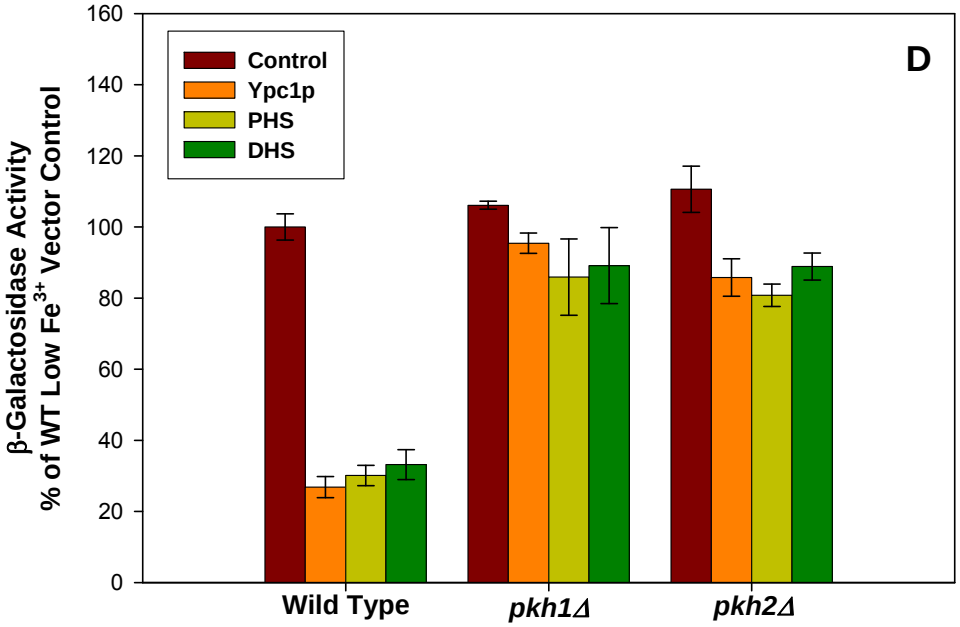

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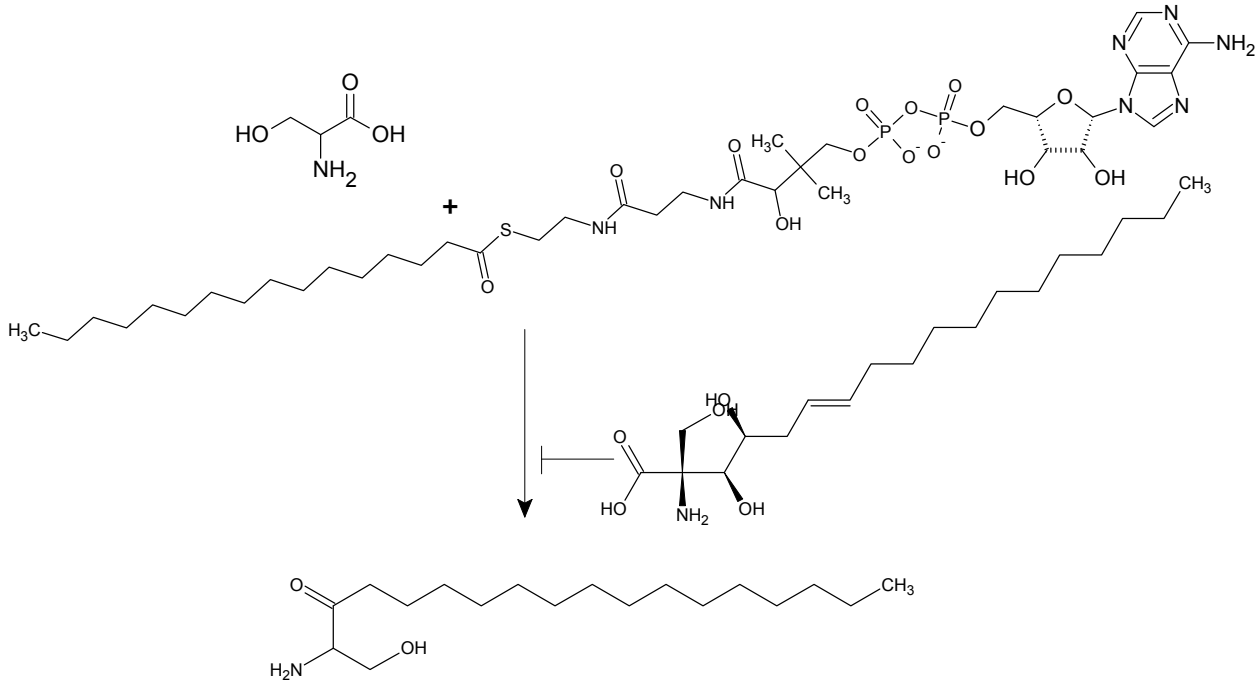
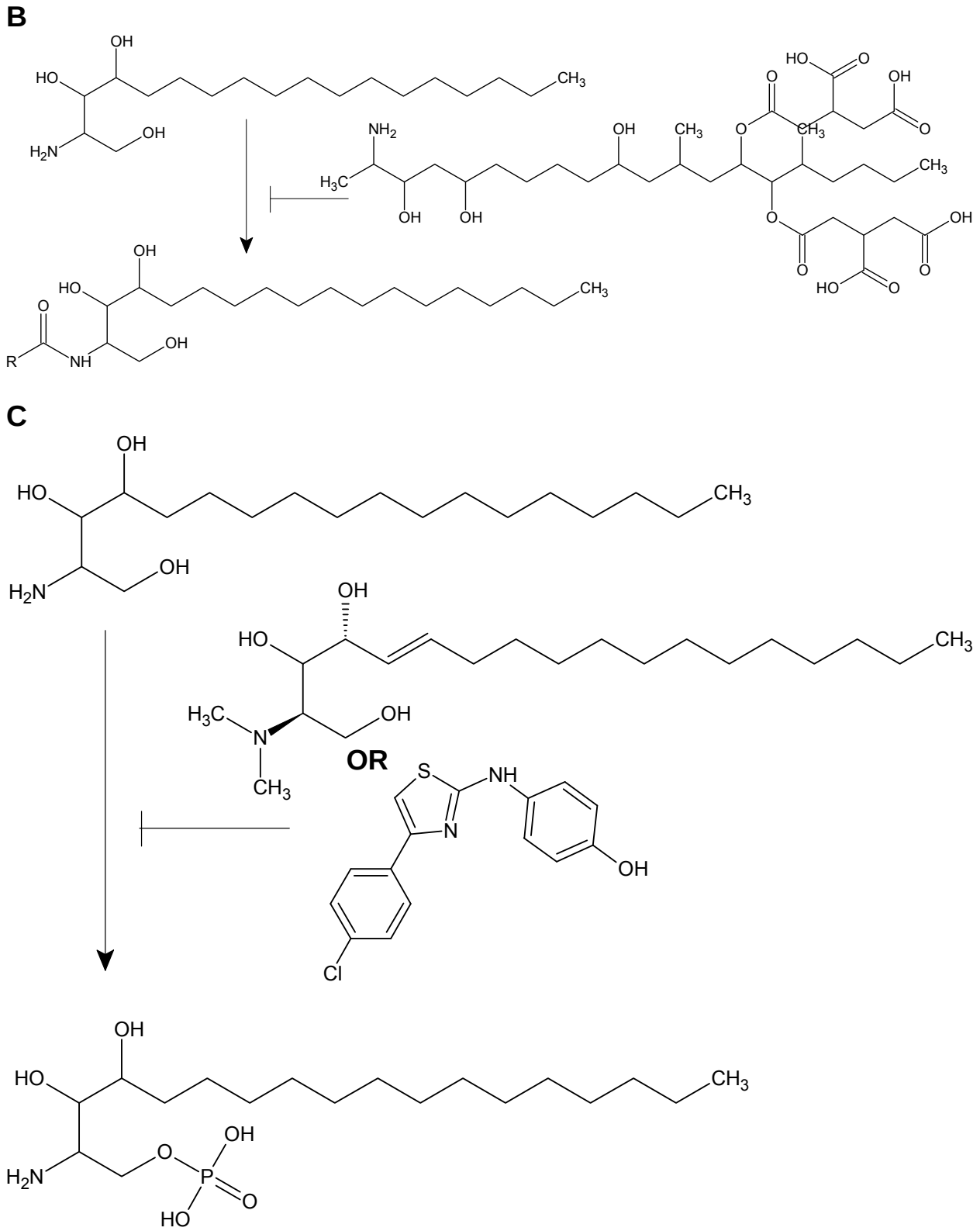


Figure 4-6. Inhibitors of sphingolipid synthesis and breakdown. (A) Myriocin inhibits the formation of 3-ketodihydrosphingosine from palmityl-CoA and L-serine. (B) Fumonisin B_1 inhibits the formation of phytoceramide from phytosphingosine. (C) N,N-dimethylsphingosine or sphingosine kinase inhibitor 1 inhibits the formation of dihydrosphingosine-1-phosphate or phytosphingosine-1-phosphate from dihydrosphingosine or phytosphingosine. Dihydrosphingosine-1-phosphate or phytosphingosine-1-phosphate is broken down to palmityl-aldehyde and phosphoryl-ethanolamine.



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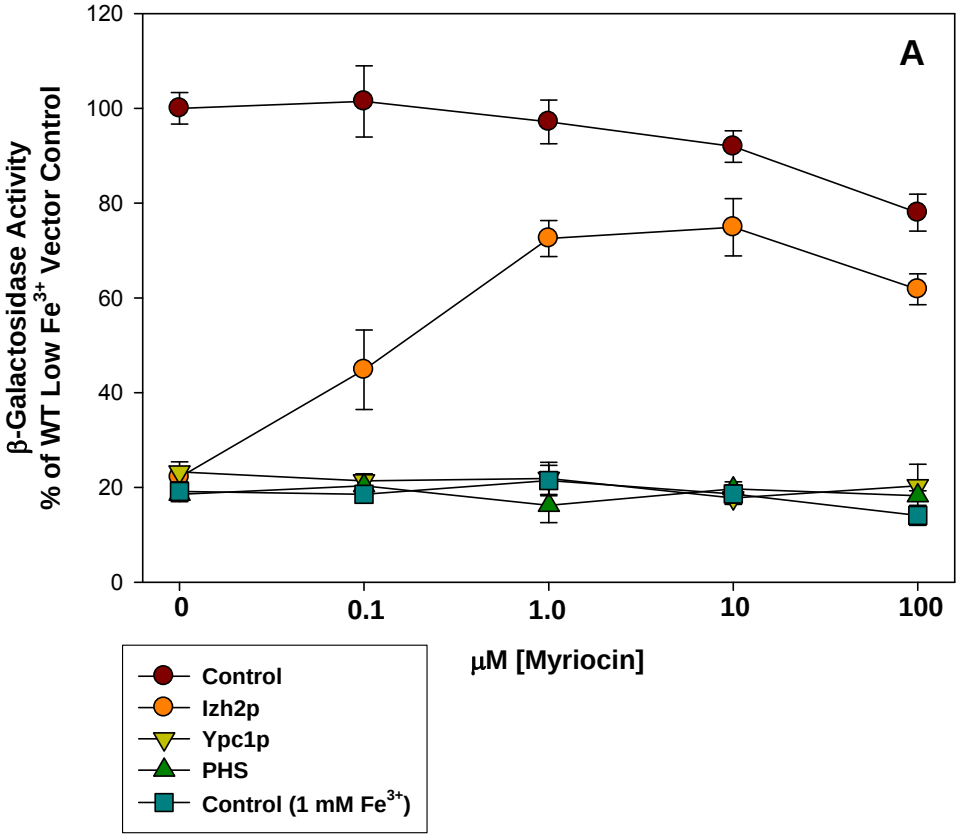
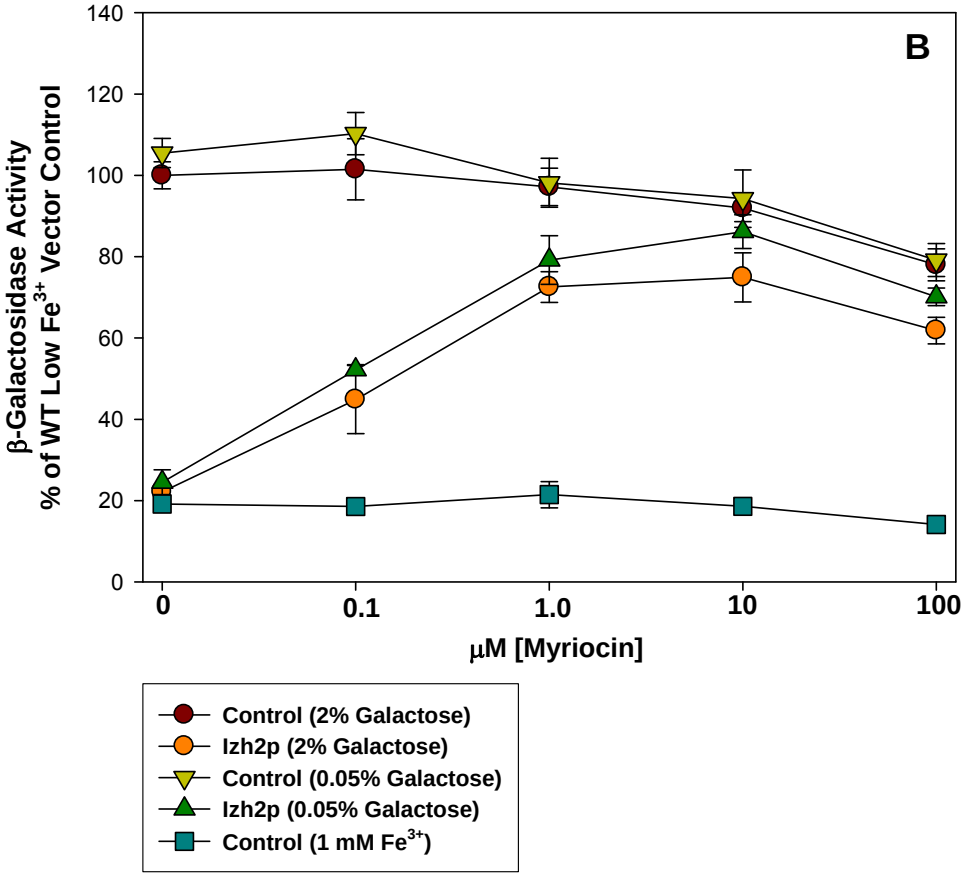


Figure 4-7. *IZH* overexpression is affecting sphingoid metabolism. BY4742 wild type strain is used in panels A to C. β-Galactosidase (Panel A to C) activities are shown as a percentage of fully induced β-galactosidase activity in wild type strain carrying an empty control vector grown in iron-deficient or iron-replete LIM. The error bars represent ± 1 standard deviation for experiments performed in triplicate (A) Myriocin alleviates repression of p*FET3*-lacZ in BY4742 wild type strain carrying the IZH2 overexpressor, but not when *YPC1* is overexpressed or phytosphingosine is added. (B) Myriocin alleviates repression of p*FET3-lacZ* due to the activation of Izh2p by thaumatin in 0.05% galactose iron-deficient LIM. (C) N,N-dimethylsphingosine (N,N-DMS), fumonisin B1 (FB1), and sphingosine kinase inhibitor 1 (SPH KI) repressed p*FET3-lacZ* in the BY4742 wild type strain carrying the empty vector but was not affected by *IZH2* overexpression.



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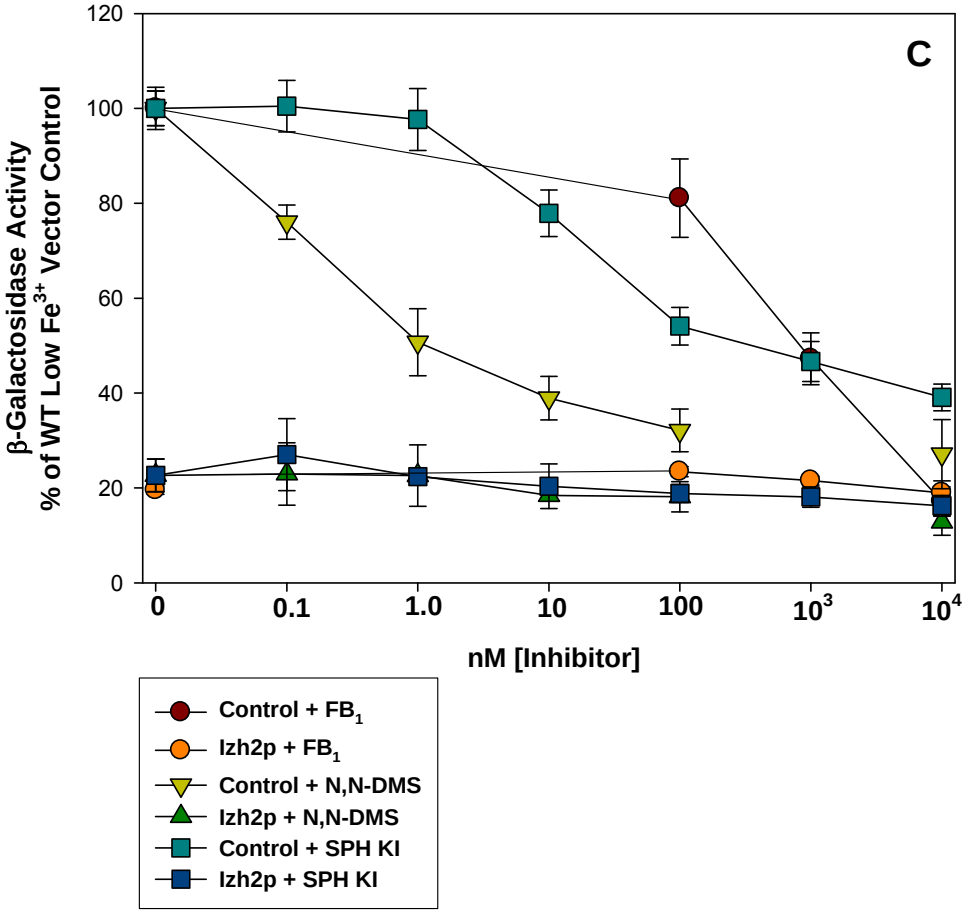

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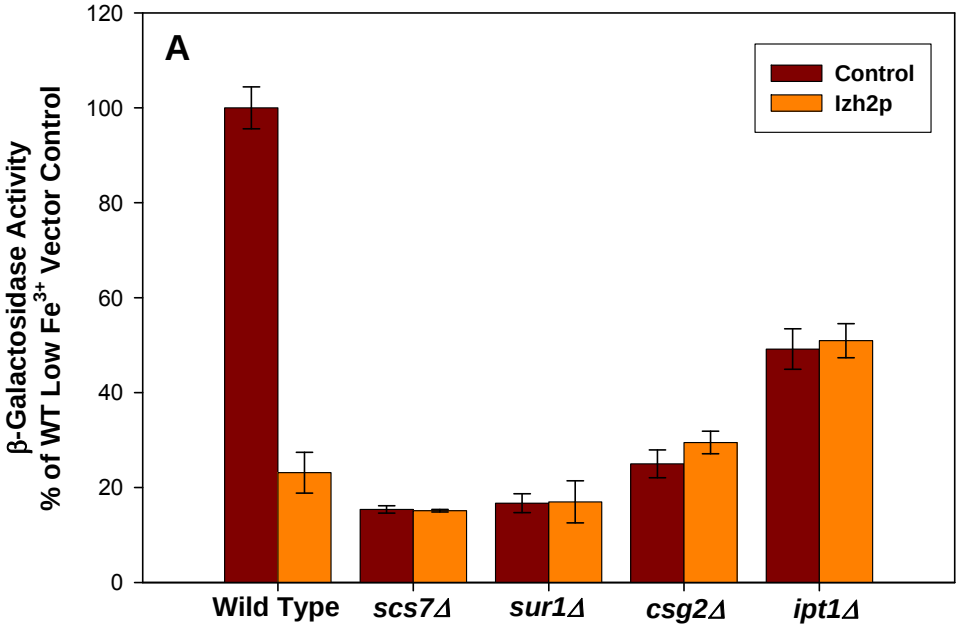
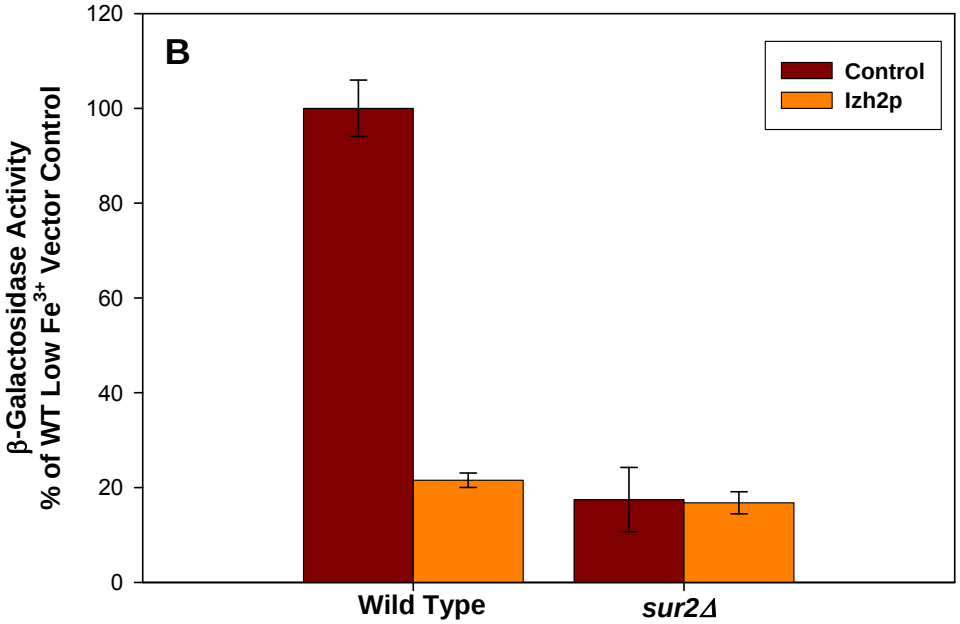
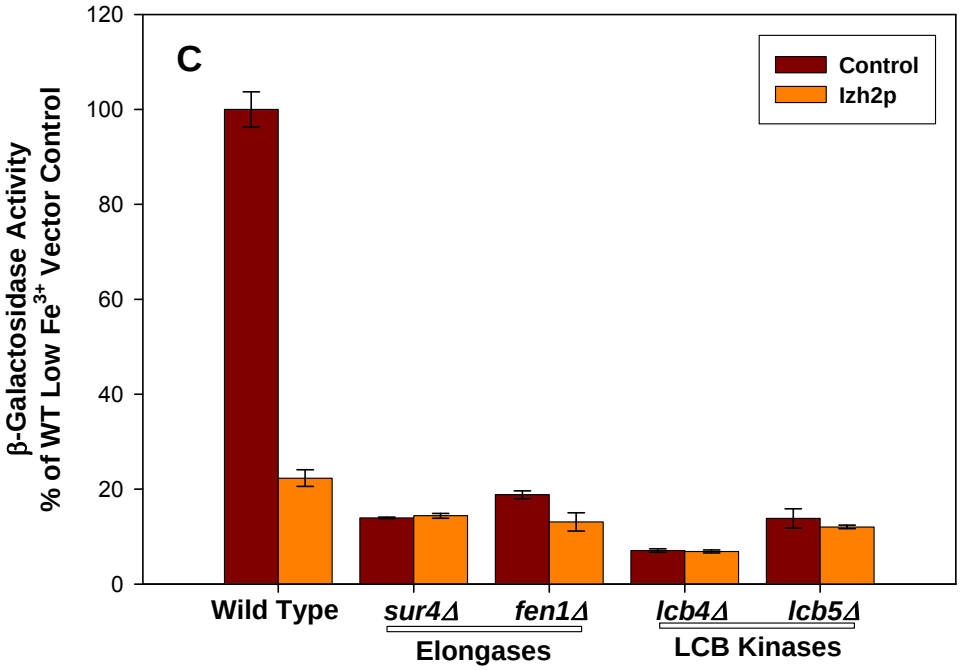


Figure 4-8. Accumulation of sphingoid bases effects *FET3*. BY4742 wild type strain is used in panels A to C. β-Galactosidase activities are shown as a percentage of fully induced β-galactosidase activity in wild type strain carrying an empty control vector grown in iron-deficient or iron-replete LIM. The error bars represent ± 1 standard deviation for experiments performed in triplicate. (A) Deletion of *SCS7*, *SUR1*, *CSG2*, and *IPT1* carrying the empty vector constitutively repressed *pFET3-lacZ*. (B) Deletion of *SUR2* carrying the empty vector constitutively repressed *pFET3-lacZ*. (C) Deletion of *LCB4*, *LCB5*, *SUR4* and *FEN1* carrying the empty vector constitutively repressed p*FET3-lacZ*.





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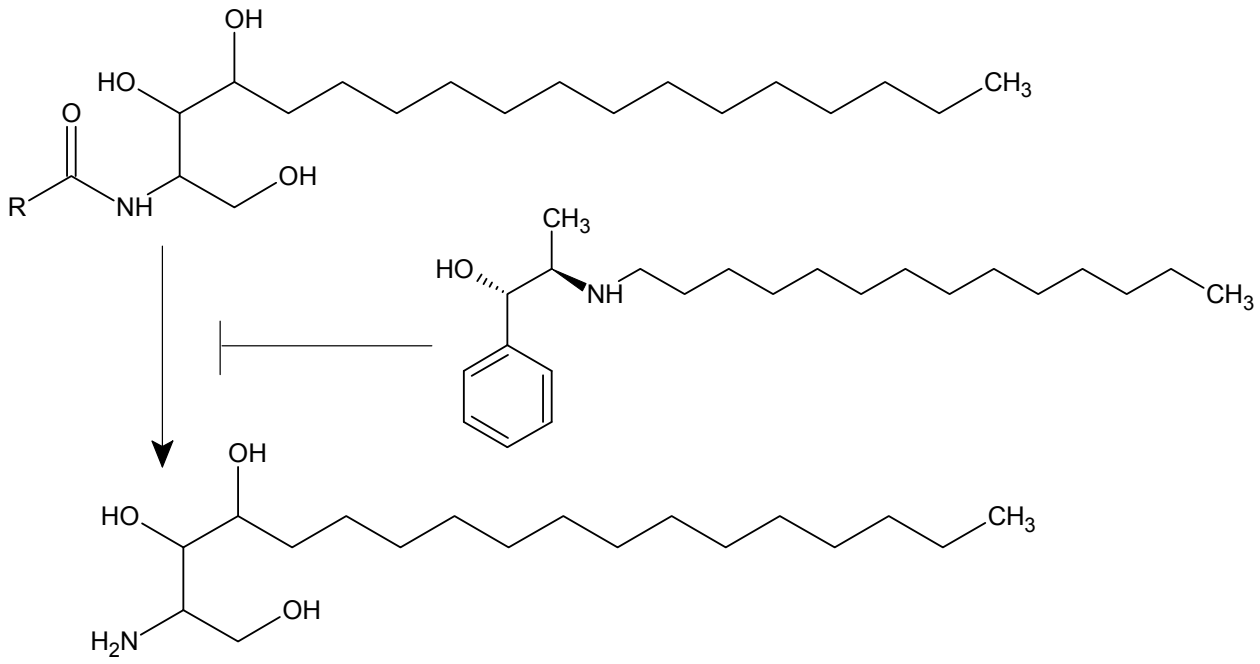


Figure 4-9. Inhibitor of ceramide breakdown. (A) D-erythro-MAPP inhibits the breakdown of phytoceramide to phytosphingosine.

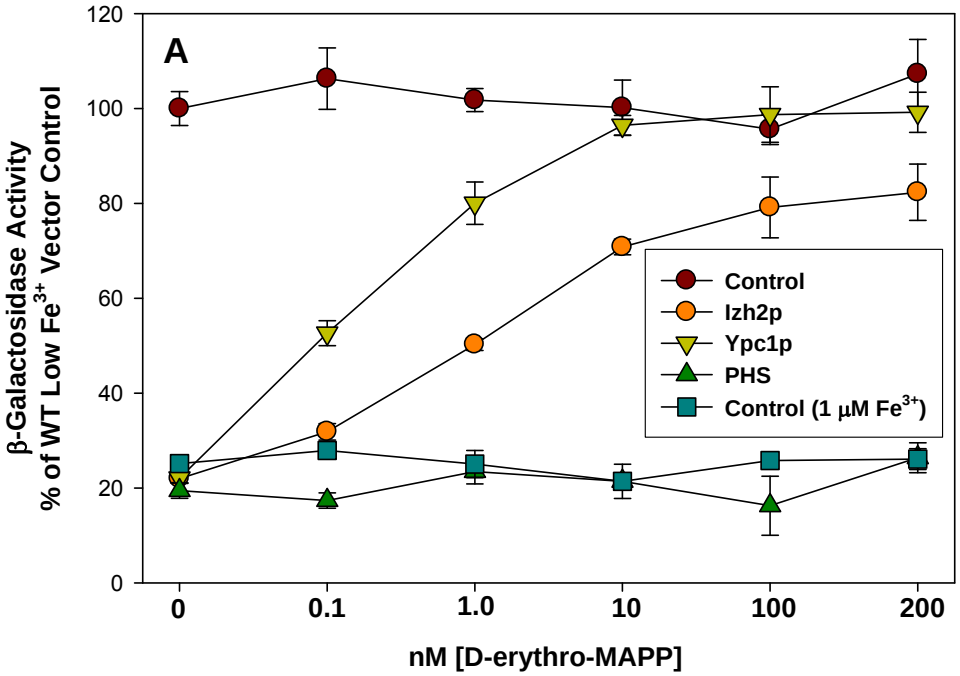
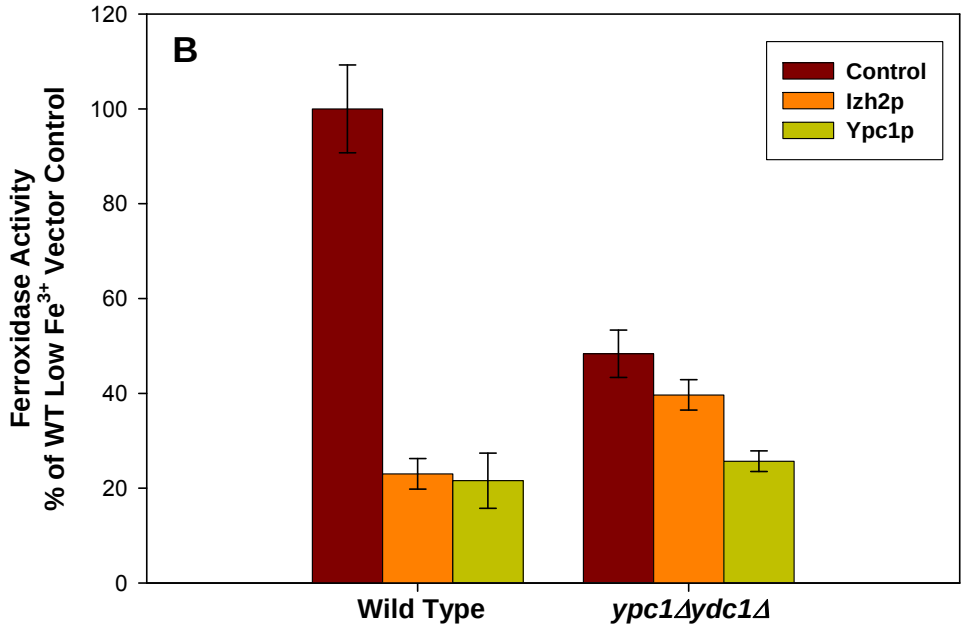
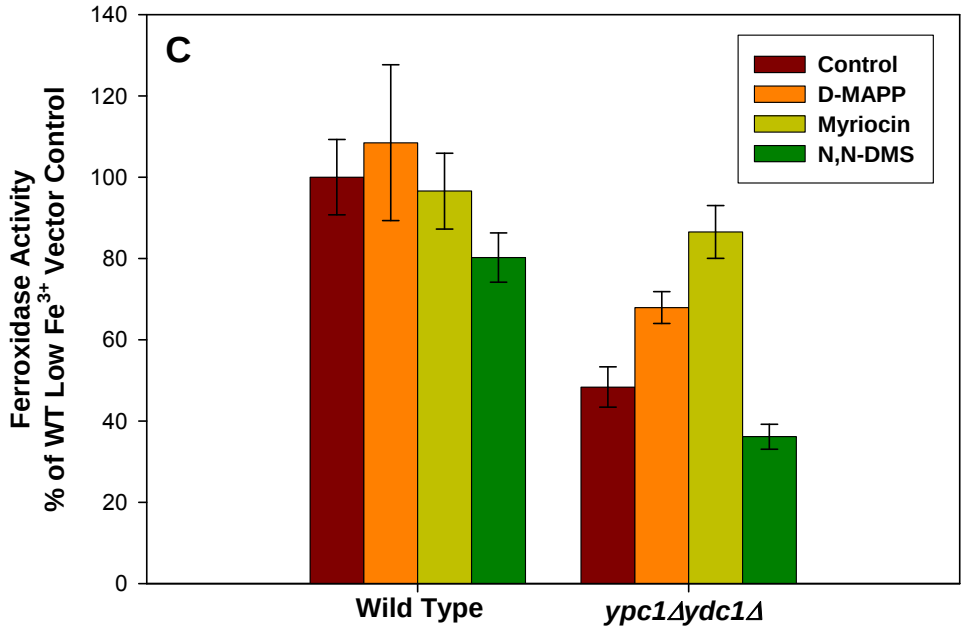


Figure 4-10. Involvement of alkaline ceramidases in Izh2p-dependent signaling. BY4742 wild type strain is used in panels A to C, while *ypc1Δydc1Δ* was used in panels B and C. β-Galactosidase (Panel A) and ferroxidase (Panel B and C) activities are shown as a percentage of fully induced β-galactosidase and ferroxidase activity in wild type strain carrying an empty control vector grown in iron-deficient or iron-replete LIM. The error bars represent ± 1 standard deviation for experiments performed in triplicate. (A) D-*erythro*-MAPP (D-MAPP) alleviates repression of p*FET3-lacZ* in BY4742 wild type strain carrying the *IZH2* or *YPC1* overexpressor, but not when phytosphingosine is added. (B) Deletion of *YPC1YDC1* carrying the empty vector causes partial repression of ferroxidase activity, while *IZH2* overexpression only causes a slight repression in this strain. Ypc1p overexpression restored repression of p*FET3-lacZ* in the *ypcΔydcΔ* mutant strain (C) Ferroxidase activity in *ypc1Δydc1Δ* mutant strain carrying the empty vector is alleviated by the addition of D-*erythro*-MAPP or myriocin, but is not effected by N,N-DMS.





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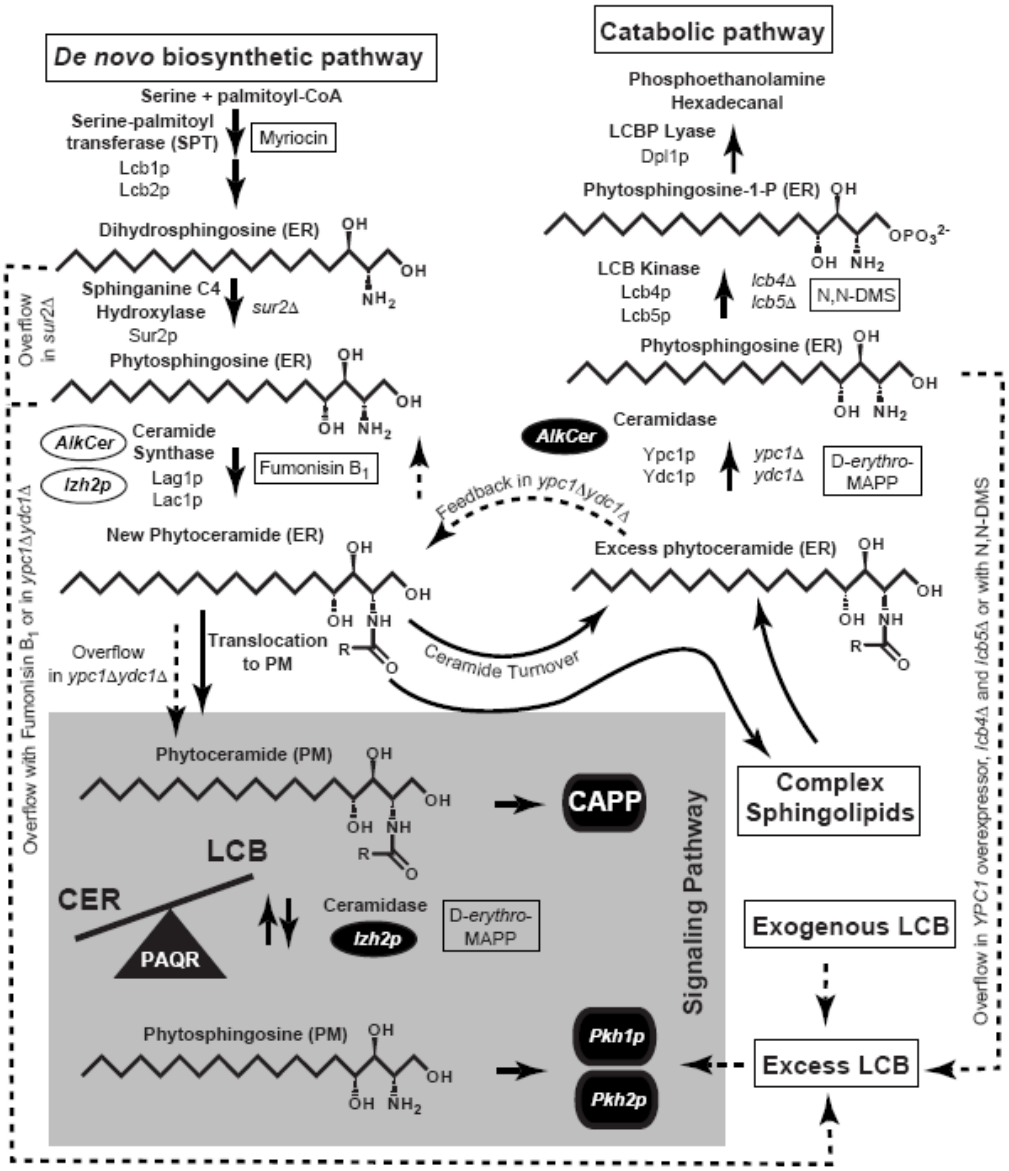


Figure 4-11. Role of sphingolipids in *IZH*-dependent signaling of *FET3*. Sphingolipids are made in the endoplasmic reticulum (ER) and migrates to the plasma membrane (PM) where they are involved in a variety of roles in yeast. Complex sphingolipids function on the cell surface while ceramidases and sphingoid bases have roles in signal transduction. Excess sphingolipids are degraded by a catabolic pathway. Ovals indicate the known sites of alkaline ceramidases activity and proposed sites of Izh2p action. Boxes indicate sites of inhibitor action. Dotted arrows indicate mechanisms by which elevation of sphingoid base may result. CAPP = **C**eramide **A**ctivated **P**rotein **P**hosphatase. In yeast nomenclature a gene IZH2 encodes a protein called Izh2p can be deleted to make a knockout strain referred to as *izh2Δ*.

CHAPTER 5
PAQRS REPRESS *FET3* IN SIMILAR MANNER AS *IZH2*

Introduction

The reason for this study is data indicating that integral membrane protein receptors, which belong to the recently discovered PAQR family of receptors, are involved in regulating high affinity iron-uptake, which belong to the recently discovered PAQR family of receptors. The human PAQR family consists of eleven proteins, which can be grouped into three classes based on sequence similarity and structural features. The human genome encodes four Class I receptors (AdipoR1, AdipoR2, PAQR3, and PAQR4), which are distinguished by seven transmembrane domains. Two of these proteins are adiponectin receptors, AdipoR1 and AdipoR2, which bind to its ligand adiponectin. Adiponectin is a hormone that has an insulin-sensitizing anti-diabetic effect and is a potential link between obesity and type II diabetes (Yamauchi et al., 2003). AdipoR1 and AdipoR2 have a confirmed topology with the C-terminus found on the outside of the plasma membrane (Kim et al., 2003; Yamauchi et al., 2003).

The Class II clade consists of five proteins (mPRα, mPRβ, mPRγ, PAQR6, and PAQR9) (Zhu et al., 2003a). Three of these proteins, mPRα, mPRβ, and mPRγ, bind to the steroid hormone, progesterone, which has many roles in reproduction, sexual behavior, ovulation and pregnancy (Mulac-Jericevic et al., 2000; Conneely et al., 2002; Mulac-Jericevic and Conneely, 2004). Due to its numerous functions, progesterone and its related derivatives have an array of medical uses ranging from birth control to breast cancer treatment (Sitruk-Ware and Plu-Bureau, 1999). Unlike Class I receptors, Class II receptors are predicted to have an eighth transmembrane domain on the C-terminus. Two more proteins in this family have also been identified, PAQR10 and PAQR11, which are highly divergent and are more closely related to the bacterial hemolysin III than the other PAQRs (Chen et al., 2004). These proteins make up the

Class III PAQRs. The presense of three highly conserved motifs, which are explained in chapter 1, are found on the cytoplasmic side of the membrane enables us to unify the three classes of human PAQRs (Lyons et al., 2004).

Saccharomyces cerevisiae also possesses four Class I PAQR receptors named Izh1p-4p (Lyons et al., 2004). One of these receptors, Izh2p, is a receptor for osmotin, a plant defensin, that is structurally similar to adiponectin. Osmotin also has been shown to activate adiponectin receptors, which suggests there is conservation of function (Narasimhan et al, 2005), Therefore, yeast is an excellent model for the study of PAQR receptors.

We chose yeast as a model system for three reasons: 1) Yeast is a eukaryotic system and its simplicity allows the user to elucidate a variety of basic biological processes. 2) *S. cerevisiae* contain receptors in the PAQR family suggesting that the parts required to interpet second messenger signals produced by these proteins is present (Lyons et al., 2004). 3) Yeast does not produce adiponectin or progesterone, and therefore, do not have have receptors for these ligands. This feature allows the user to investigate the activity of a specific human receptor of interest.

We show that the PAQRs result in repression, in most cases, of the gene encoding *FET3*. To examine this principle, we used β-galactosidase and ferroxidase assays to demonstrate that PAQR overexpression from the three classes results in repression of the *FET3* gene under conditions of iron deficiency in the same manner as Izh2p. However, AdipoR2 and mPRα were not capable of signaling repression when overexpressed. However, if their respective activating ligands were added to the growth medium, (adiponectin for AdipoR2 or progesterone for mPRα) repression of *FET3* was observed.

In this study, we show that the human PAQRs regulate the Nrg1p/Nrg2p repressors. Furthermore, we demonstrate that the human PAQRs, like the yeast PAQRs, require cAMP

dependent kinase (protein kinase A, PKA), AMP-dependent kinase (AMPK), and the sphingoid base-sensing kinases Pkh1p and Pkh2p (Kupchak et al., 2007). Thus we report that the PAQRs demonstrate a negative effect on *FET3*, and this effect occurs using a similar mechanism in which the yeast Izh2p represses *FET3*.

Materials and Methods

Yeast Strains and Plasmids

Wild type (BY4742) and mutant strains in this background (*pkh1Δ*, *pkh2Δ*, *ras2Δ*, *tpk1Δ*, *tpk2Δ*, *tpk3Δ*, *snf1Δ*, *snf4Δ*, *gal83Δ*, *sip1Δ*, *sip2Δ*, *nrg1Δ*, *nrg2Δ*, *msn2Δ*, and *msn4Δ*) were purchased from Euroscarf (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/col_index.html) and their genotypes are listed in Supplementary Table 1. The p*FET3-lacZ* plasmid (Gift of D. Eide) has the promoter region of the *FET3* gene fused to the *lacZ* open reading frame and is *URA3* selectable. PAQR proteins (PAQR3, PAQR4, mPRγ, mPRα, and PAQR11) were cloned from cDNAs into the *LEU2* selectable pRS316-*GAL1* plasmid (Lyons et al., 2004). The plasmid uses the *GAL1* promoter to drive galactose-inducible expression of cloned genes. The cDNAs used as templates were obtained from Open Biosystems or the NEDO Human cDNA Sequencing Project and primers used to clone PAQR3, PAQR4, mPRγ, mPRα, and PAQR11 are listed in Supplementary Table 2. The cDNA for PAQR3 had one intron that we removed by overlap extension PCR. All clones were sequenced to ensure that no errors occurred during PCR.

β –Galactosidase and Ferroxidase Assays

β -Galactosidase and ferroxidase sssays were performed as described in chapter 2 except for the addition of the following compounds to LIM. Progesterone (Sigma #P8783-1G), 17α-hydroxyprogesterone (#H572-5G), 21-hydroxyprogesterone (Sigma #D6875-500MG), 17α, 21-

hydroxyprogesterone (Sigma #R0500-1G) cortisol, and testosterone (Sigma #T1500-5G) were added to the cultures at varied concentrations.

Results

PAQRs are found in all eukaryotes ranging from *Saccharomyces cerevisiae* to *Homo sapiens*. Human PAQRs are composed of both adiponectin receptors and membrane progestin receptors. Overexpression of both yeast and human PAQRs causes repression of *FET3*. Thus, experiments were designed to test if yeast and human PAQRs signal in a similar manner to the Izh2p.

Effect of Human PAQRs on *FET3* Expression

Previous studies have shown that *IZH2* overexpression negatively affected the transcription of the *FET3* gene (Kupchak et al., 2007). Using a p*FET3-lacZ* reporter, we show that overexpression of Izh1p, Izh3p, Izh4p, AdipoR1, PAQR3, PAQR4, mPRβ, mPRγ, PAQR11, but not AdipoR2 and mPRα, results in repression of the p*FET3-lacZ* reporter construct even in iron-limiting media. To verify these results, we also measured the enzymatic activity of Fet3p to oxidize Fe^{2+} to Fe^{3+} using a ferroxidase assay (Figures 5-1A to 5-1C).

AdipoR2 and mPRα are Activated by its Putative Ligands.

As was shown in Figures 5-1A and 5-1B, the overexpression of the Class I receptor AdipoR2 and the Class II receptor mPRα were unable to repress p*FET3-lacZ*. In this study, we demonstrate that the addition of adiponectin (Figure 5-1C) for AdipoR2 and progesterone for mPRα to the iron-limiting LIM was effective at signaling p*FET3-lacZ* repression. Other steroids, such as 17α-hydroxyprogesterone, 21-hydroxyprogesterone, 17α, 21-dihydroxyprogesterone during mPRα overexpression were also able repress p*FET3-lacZ* for mPRα, but at a concentration 10-times higher than progesterone. The steroids cortisol and testosterone had no effect on p*FET3-lacZ* activity when mPRα was overexpressed (Figure 5-1D).

PAQRs affect *FET3* Expression

In chapter 2, we demonstrated that *IZH2* overexpression altered *FET3* regulation by affecting the transcriptional factors Msn2p/Msn4p and Nrg1p/Nrg2p. In this study, we demonstrated that Nrg1p and Nrg2p are also necessary for signal transduction of *FET3* by the yeast and human PAQRs. PAQR dependent p*FET3-lacZ* repression was lost in *nrg1Δ* and *nrg2Δ* mutant strains (Figures 5-3A to 5-3C).

PKA is known to function downstream of Izh2p (Kupchak et al., 2007). Therefore, we tested its role in PAQR-dependent repression of *FET3*. p*FET3-lacZ* activity exhibited loss of repression during PAQR overexpression in mutants lacking each of the three catalytic subunits, *tpk1Δ*, *tpk2Δ*, and *tpk3Δ* (Figures 5-5A to 5-5C). Upstream of PKA is Ras2p-cAMP, which is known to activate PKA (Jiang et al., 1998). In the *ras2Δ* mutant strain, PAQR overexpression did not cause repression of p*FET3-lacZ* (Figures 5-5A to 5-5C).

Also downstream of Izh2p is the AMPK complex (Kupchak et al., 2007). AMPK is composed of subunits that control its activity and localization. Deletion of *SNF4*, the stimulatory γ-subunit of AMPK caused partial repression of p*FET3-lacZ*, while overexpression of the PAQRs in the *snf4Δ* mutant strain had no further effect. Strains lacking AMPK's cytoplasmic β subunits, Sip1p and Sip2p, showed partial repression of p*FET3-lacZ* and also was not effected by overexpression of the PAQRs (Figures 5-3A and 5-3C). Besides AMPK β and γ subunits, there are other proteins that intereact with AMPK in yeast. One of these proteins, Sip3p, is also essential for PAQR dependent repression as there loss results in the inability to repress p*FET3-lacZ* (Figures 5-4A to 5-4C).

In this study, we show the genes *PKH1* and *PKH2* are involved PAQR-dependent *FET3* repression. Loss of Pkh1p and Pkh2p results in complete loss of p*FET3-lacZ* repression.

Although Pkh2p plays a greater role for repression when AdipoR1, AdipoR2, PAQR3, or PAQR4 are overexpressed; however, Pkh1p seems to be more important for the remaining PAQRs in repressing p*FET3-lacZ* activity (Figures 5-6A and 5-6B).

Discussion

The PAQR receptors are of critical importance to human health. First, AdipoR1 and AdipoR2 function as receptors for adiponectin, a protein secreted by adipocytes that has emerged as a candidate for the molecular link between obesity and type II diabetes (Kadowaki and Yamauchi, 2005). Second, human PAQRs also encode proteins for membrane progestin receptors, which are known to bind to progesterone (Zhu et al., 2003b). Finally, human PAQR10 and PAQR11 proteins make up the final class of receptors, which are presumed to be involved in monocyte to macrophage maturation (Bauer, et al., 2004).

Human PAQRs were expressed in the eukaryotic model organism, *Saccharomyces cerevisiae*. An important reason for this choice is that yeast do not possess adiponectin or membrane progestin receptors, thus minimizing interference with data analysis due to progesterone or adiponectin's inability to bind to other PAQR receptors.

PAQRs Repress *FET3* Expression

In previous studies, we demonstrated that Izh2p activity could be monitored by using a β-galactosidase and ferroxidase assays, and that overexpression of *IZH2* represses *FET3*. The Izh proteins belong to a larger family of membrane proteins called the PAQRs, and consist of human adiponectin and membrane progestin receptors. Like Izh2p, overexpression of the yeast (Izh1p, Izh3p, and Izh4p) and human (AdipoR1, PAQR3, and PAQR4) Class I homologues and the human Class III homologue, (PAQR11) caused *FET3* repression. AdipoR2 was unable to repress *FET3*, unless adiponectin was added to the media. Control experiments exhibited that adiponectin did not affect *FET3* in yeast carrying the empty vector, thereby indicating that

adiponectin's effect was specific for AdipoR2. Thus, human PAQR homologues can be functionally expressed in yeast. We propose that some human PAQR homologues, like many signaling proteins, have intrinsic basal signal capability that is amplified by overexpression, making the presence of an activating ligand unnecessary. AdipoR2, on the other hand, requires adiponectin for activation to signal the repression of *FET3*.

Effect of Class II Homologues on *FET3* Expression

Human Class II PAQRs, mPRα, mPRβ, and mPRγ were expressed in yeast and our experiments demonstrated that mPRβ, and mPRγ were capable of repressing *FET3*, while mPRα did not signal repression. mPRα was activated by the addition of progesterone to transmit the repression signal. The effect of steroids on mPRα is highly specific for progesterone: 17α-hydroxyprogesterone, 21-hydroxyprogesterone, and 17α,21-dihydroxyprogesterone were an order of magnitude less effective than progesterone at activation, while cortisol and testosterone were completely ineffective. Control experiments showed that the addition of progesterone did not affect *FET3* in yeast that were carrying an empty vector, indicating that progesterone does not non-specifically affect *FET3*. Therefore, we conclude that mPRβ and mPRγ are capable of basal signal transduction, while mPRα is stimulated by progesterone to affect *FET3*.

PAQRs Repress *FET3* by a Common Mechanism

All members of the human PAQRs either have basal signaling capability or can be activated to repress *FET3*. Therefore, we propose that the PAQRs have a common signaling mechanism upstream of *FET3*. This possibility is supported by several lines of evidence from the literature. First, both adiponectin (Xi et al., 2005) and progesterone (Alkhalaf et al., 2002) have been reported to activate PDK (Vanhaesebroeck and Alessi, 2000). The requirement for progesterone can be seen in the re-initiation of meiosis in *Xenopus* oocytes, an event that requires mPRs, and can be bypassed by the addition of the PDK activator sphingosine (Strum et al.,

1995). Also, PDK has been shown to play an important part in insulin signaling (Mora et al., 2005), a process that requires adiponectin. Therefore we investigated if Pkh1p and Pkh2p were required for PAQR-dependent repression of *FET3*. Similar to Izh2p, the PAQRs require both Pkh1p and Pkh2p to repress *FET3*.

Previous studies have also shown the effects of adiponectin and progesterone are alleviated by inhibiting PKA activity (Kline and Karpinski, 2005) (Ouchi et al., 2000). Therefore, we investigated if the PKA's subunits were necessary for signal transduction of *FET3* repression. Indeed, *TPK1*, *TPK2*, *TPK3* and *RAS2* were all needed for *FET3* repression to occur.

Based on previous reports in our laboratory, we also demonstated the AMPK complex was necessary for signal transduction. Our results were verified by studies in which human AdipoR1 signals downstream through AMPK (Yamauchi et al., 2003). Our studies also demonstrated that *FET3* repression requires the AMPK β-subunits (Sip1p, and Sip2p), and γ-subunit (Snf4p) as shown in chapter 3. Also, a cytolasmic Snf1p interacting protein, Sip3p, is required for repression (Lesage, et al., 1994). This is intriguing since its human homologue, APPL1, is required for signal transduction by AdipoR1 (Mao et al., 2006).

Even though the effect on *FET3* by yeast PAQR requires Nrg1p/Nrg2p for repression and Msn2p/Msn4p for activation (Kupchak et al., 2007), there was no evidence in the literature to claim these transcription factors were required for PAQR-dependent repression of *FET3*. We proceeded to test human PAQRs effect on *FET3* in knockout strains of both Nrg1p/Nrg2p, and indeed, the effect of the PAQRs on *FET3* did require both Nrg1p/Nrg2p.

Thus, *FET3* repression by the human PAQRs requires Pkh1p, Pkh2p, Ras2p, Tpk1p, Tpk2p, Tpk3p, Sip1p, Sip2p, Snf1p, Snf4p, Nrg1p, Nrg2p, Msn2p, and Msn4p in yeast (Kupchak et al., 2007) and that adiponectin and progesterone have been shown to regulate some

of these proteins in vertebrate cells. This does not necessarily mean an identical signaling mechanism exists in vertabrates; however, our data support a model where the human PAQRs repress *FET3* in yeast through a common mechanism.

Summation

Our findings support several important conclusions. First, every human PAQR tested, regardless of activating ligand and physiological function affected *FET3*. Second, we have presented data exhibiting that AdipoR2 and mPRα senses and responds to its particular ligand when expressed in yeast cells. Third, the human PAQRs signal *FET3* repression in a similar mechanism as the yeast PAQRs.

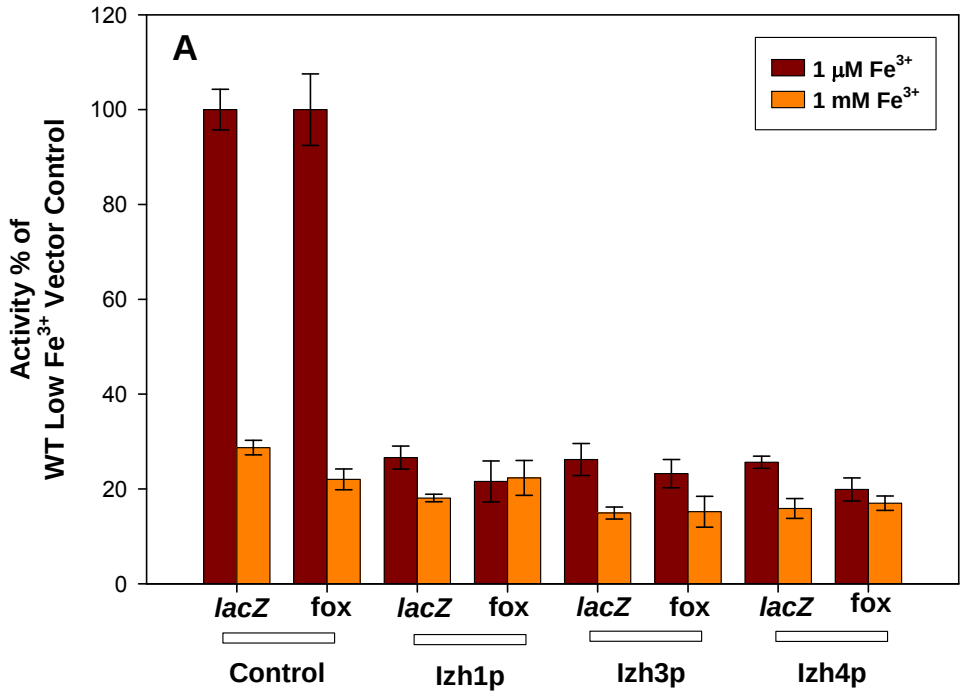
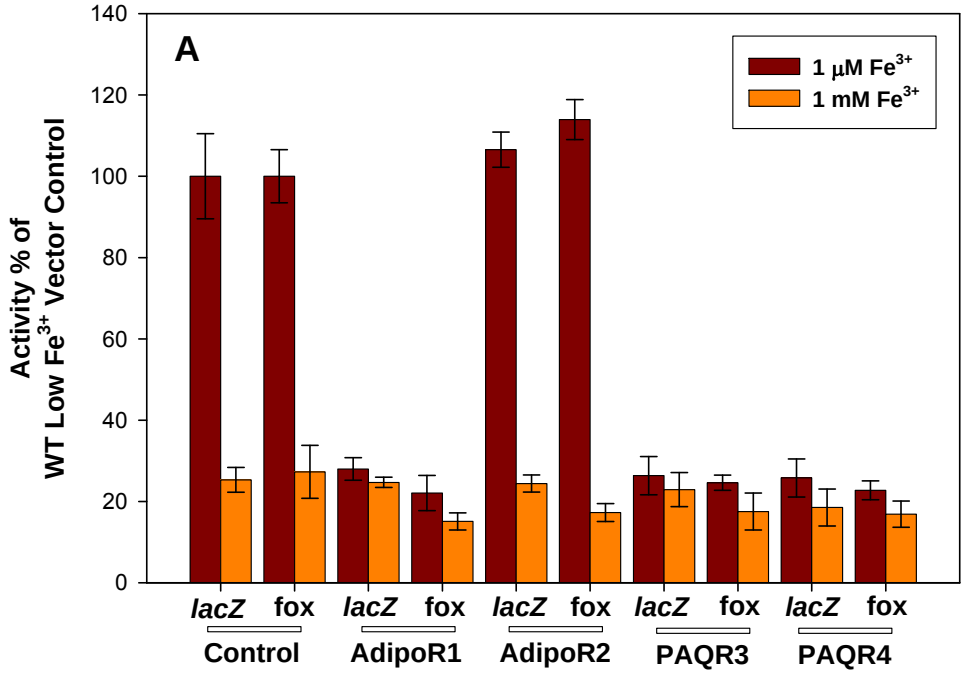
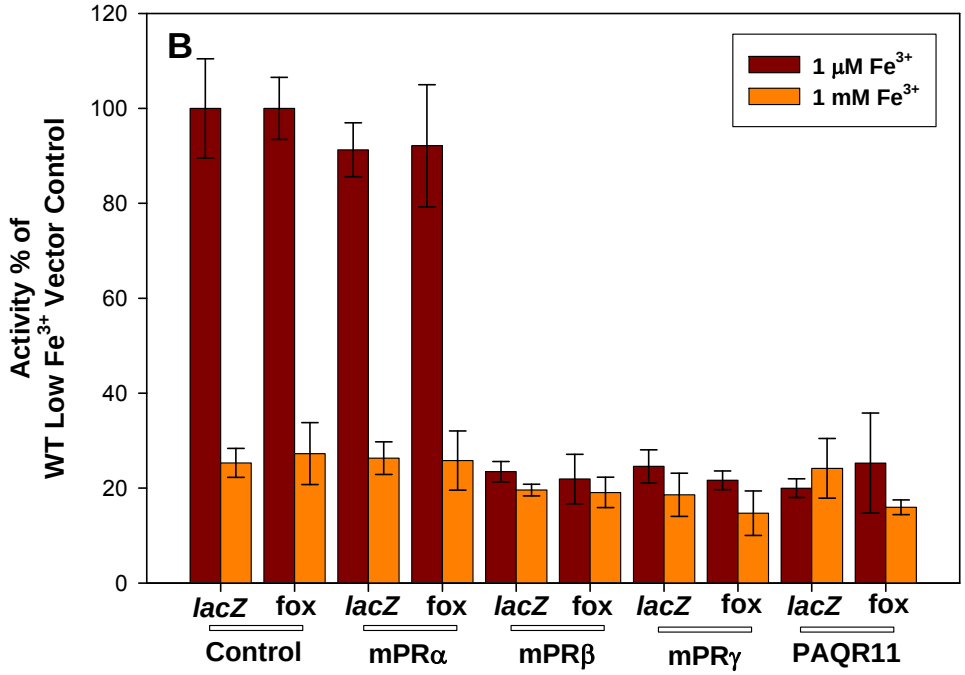


Figure 5-1. Class I, II, and III PAQRs repress *FET3* transcription**:** BY4742 wild type strain is used in panels A, B and C. β-Galactosidase (Panels A, B, and C) and ferroxidase (Panel A) activities are shown as a percentage of fully induced activity in wild type strain carrying an empty control vector grown in iron-deficient and iron-replete LIM. The error bars represent ± 1 standard deviation for experiments performed in triplicate. (B) β-Galactosidase activity of the p*FET3-lacZ* reporter and the cell surface ferroxidase activity are induced in iron-deficient LIM in the wild type strain carrying the AdipoR1, PAQR3, PAQR4, mPRβ, mPRγ, and PAQR11 overexpressor, but not the AdipoR2 and mPRα overexpressor. (D) AdipoR2 expression in 2% galactose has no effect on p*FET3-lacZ* unless the ligand adiponectin is added to iron-deficient LIM. (E) mPRα expression in 2% galactose has no effect on p*FET3-lacZ* unless a ligand is added to iron-deficient LIM. Progesterone (PG), 17α-hydroxyprogesterone (17α-HPG), 21-hydroxyprogesterone (21-HPG), and 17α,21-hydroxyprogesterone (17α,21-DHPG) exhibited a repression of p*FET3-lacZ*, while cortisol and testosterone (Test) had no effect on p*FET3-lacZ* repression.





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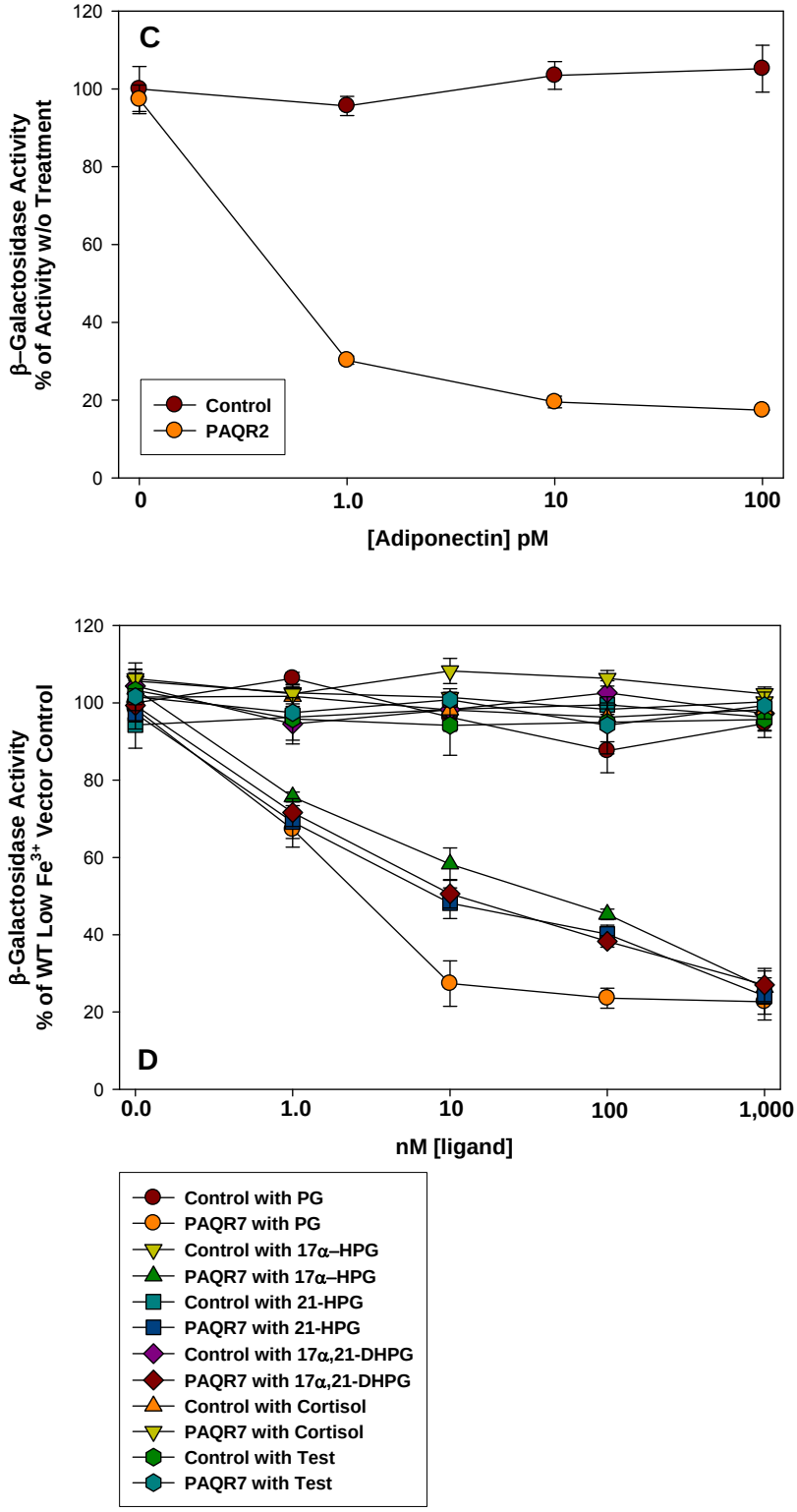

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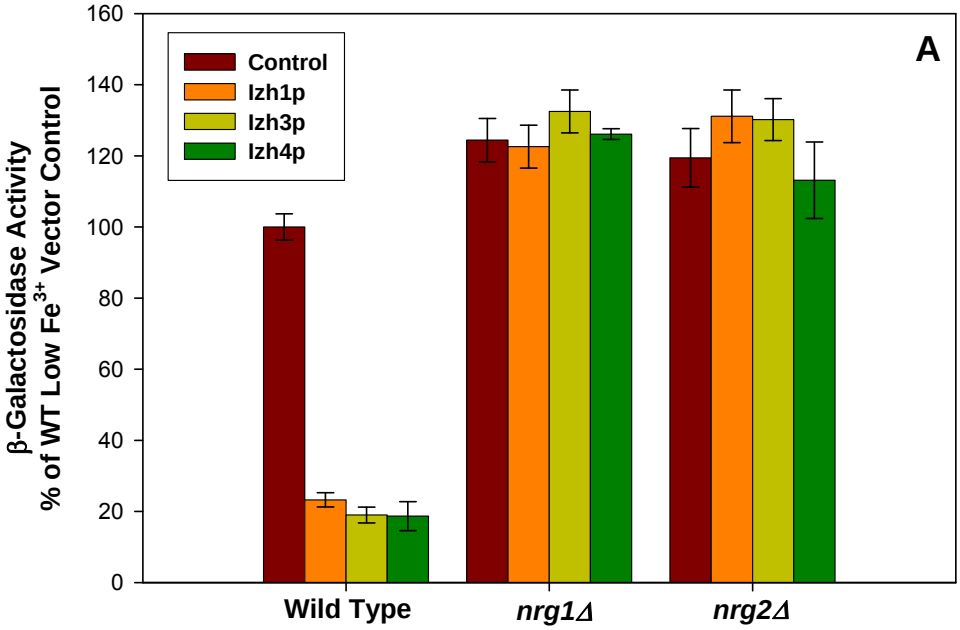
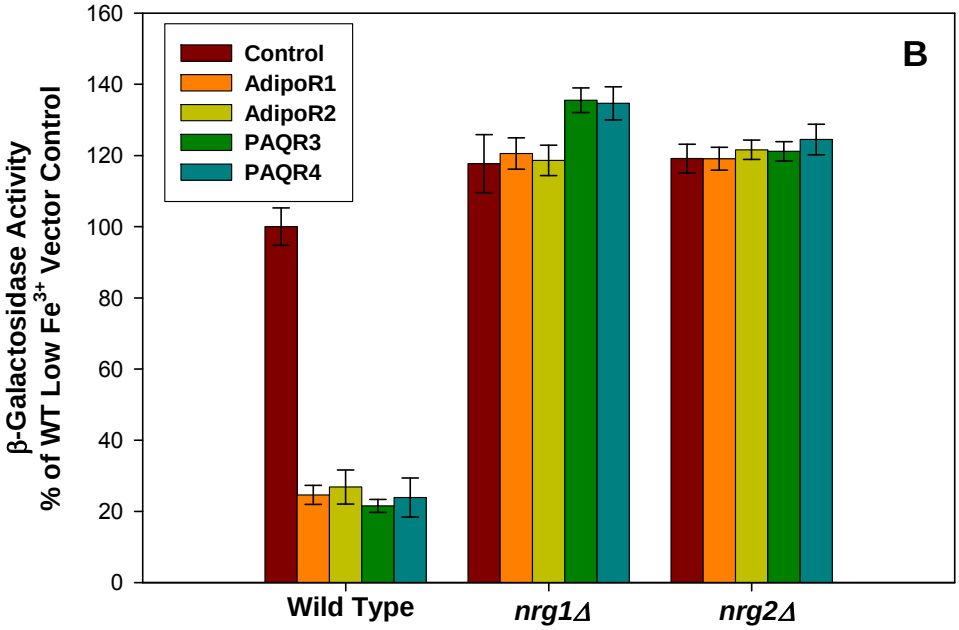
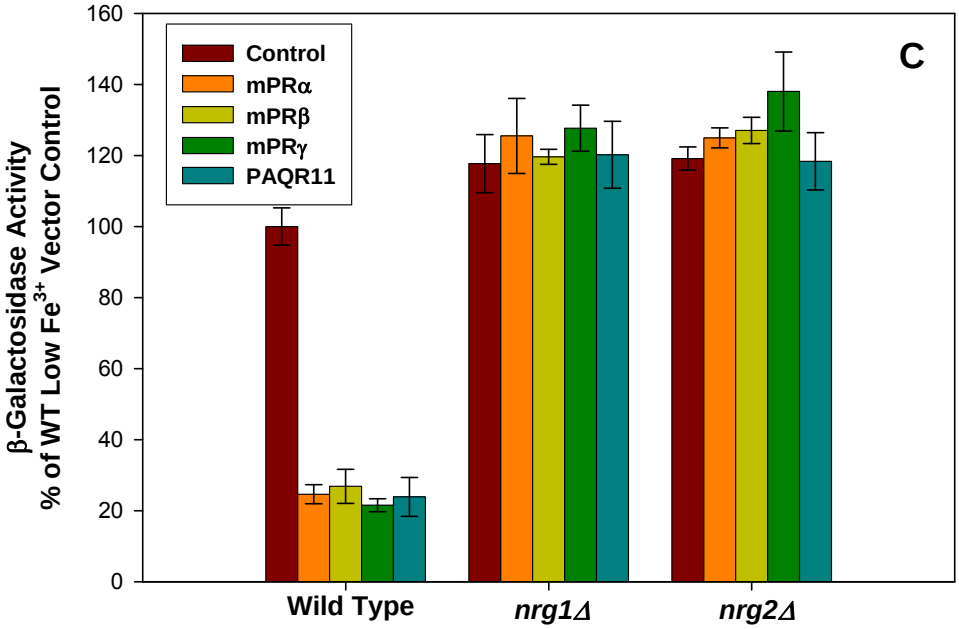


Figure 5-2. Nrg1p and Nrg2p effect on PAQR-dependent *FET3* repression: BY4742 wild type, *nrg1Δ*, and *nrg2Δ* strains are used in panels A and B. β-Galactosidase activities are shown as a percentage of fully induced activity in wild type strain carrying an empty control vector grown in iron-deficient and iron-replete LIM. The error bars represent ± 1 standard deviation for experiments performed in triplicate. (A) The repression of β-galactosidase activities of the p*FET3-lacZ* reporter by overexpression of Izh1p, Izh3p, and Izh4p were absent in the *nrg1Δ* and *nrg2Δ* mutant strains. (B) The repression of β-galactosidase activities of the p*FET3-lacZ* reporter by overexpression of AdipoR1, AdipoR2 with adiponectin, PAQR3, and PAQR4 were absent in the *nrg1Δ* and *nrg2Δ* mutant strains. (C) The repression of β-galactosidase activities of the p*FET3-lacZ* reporter by overexpression of mPRα with progesterone, mPRβ, mPRγ, and PAQR11 were absent in the *nrg1Δ* and *nrg2Δ* mutant strains.





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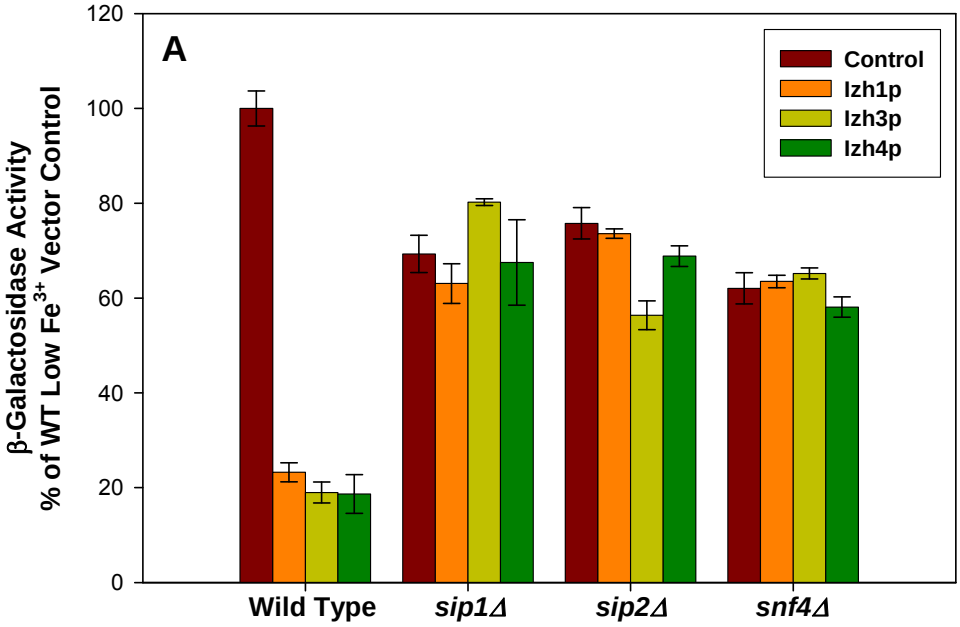
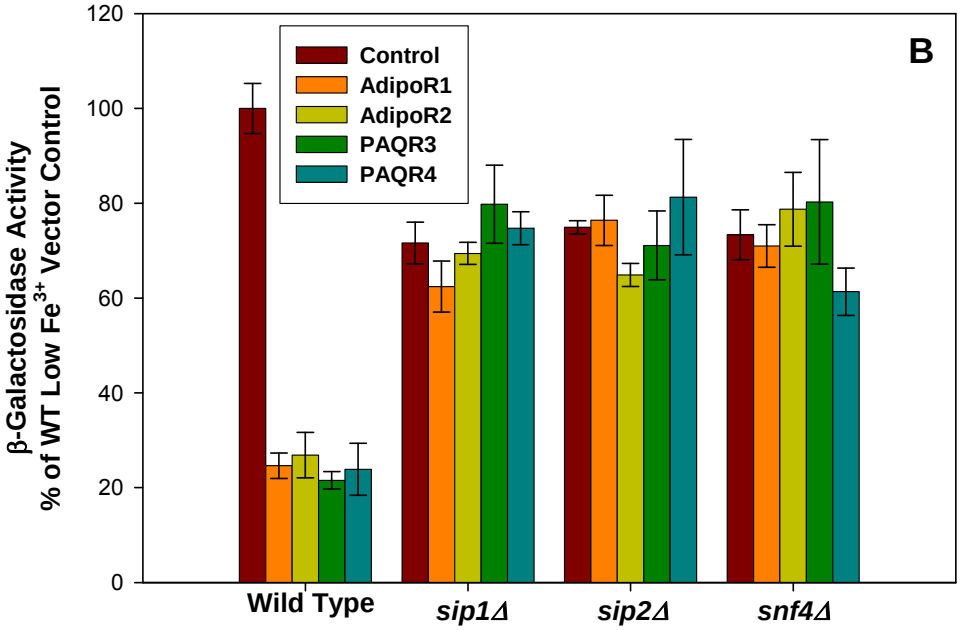
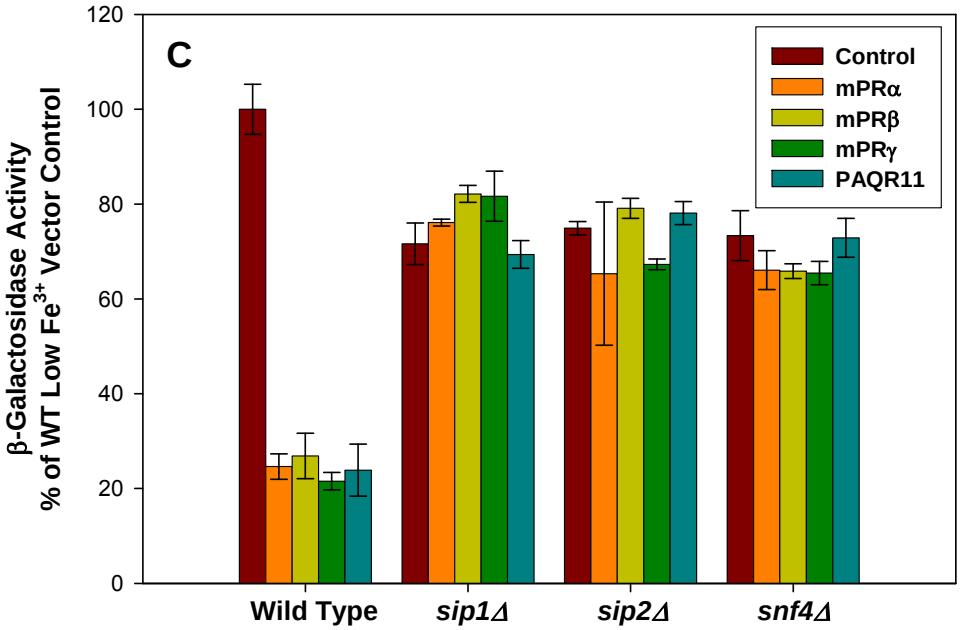


Figure 5-3: AMPK in *FET3* repression: BY4742 wild type strain is used in panels A, B, C and D. *snf4Δ*, *sip1Δ*, and *sip2Δ* strains are used in panels A to C. β-Galactosidase activities are shown as a percentage of fully induced activity in wild type strain carrying an empty control vector grown in iron-deficient and iron-replete LIM. The error bars represent ± 1 standard deviation for experiments performed in triplicate. (A to C) β-galactosidase activities of the p*FET3-lacZ* reporter is partially repressed in *snf4Δ*, *sip1Δ*, and *sip2Δ* mutant strains and PAQR overexpression has no effect on activity.





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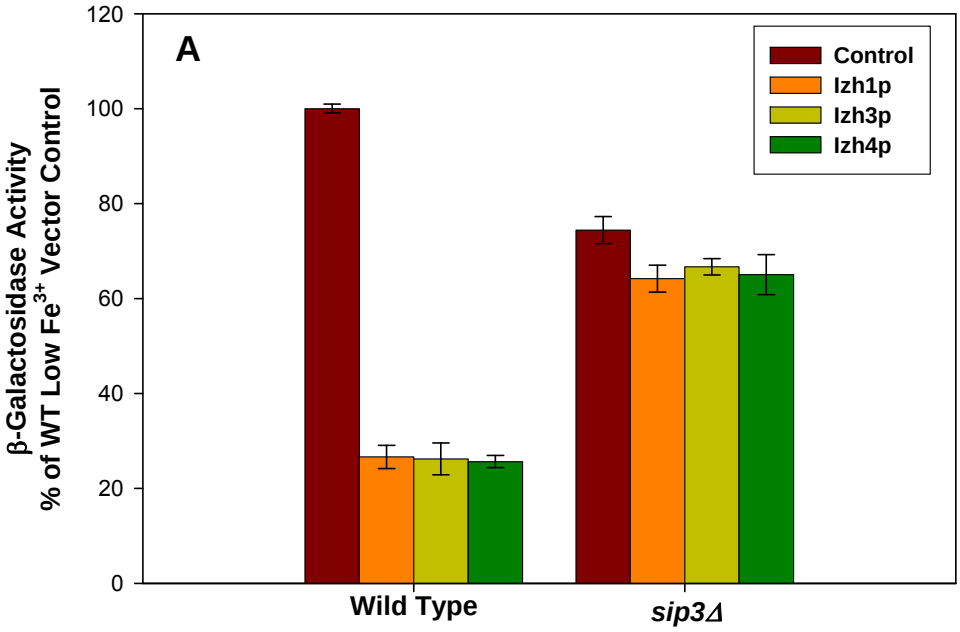
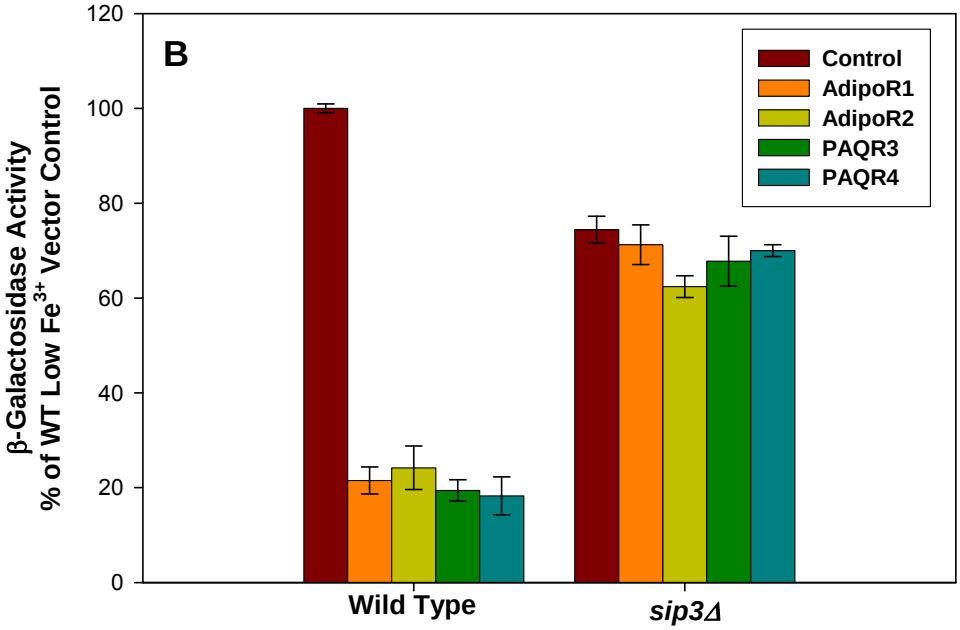
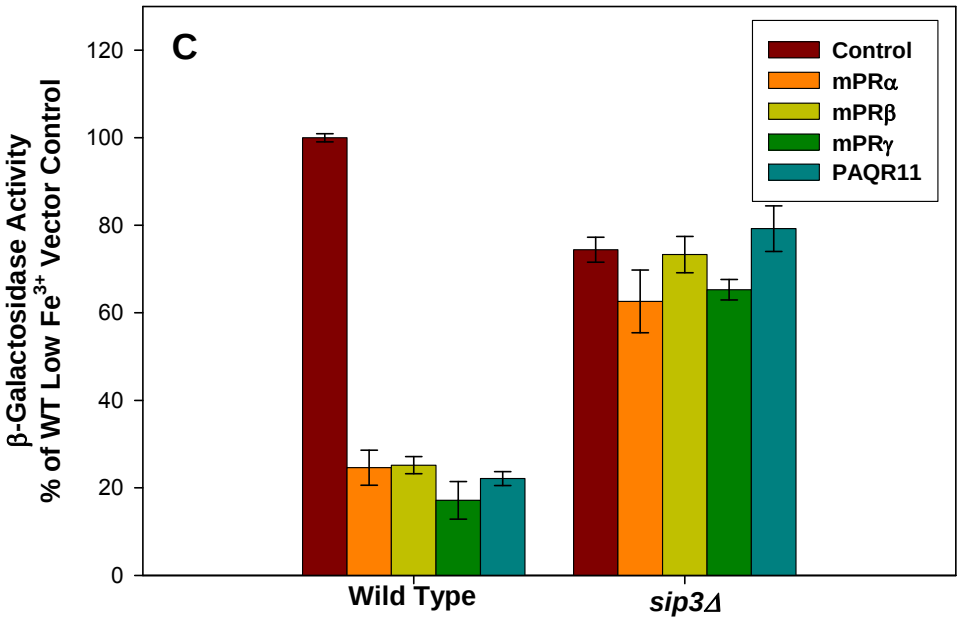


Figure 5-4. Sip3p in *FET3* repression: BY4742 wild type strain is used in panels A, B and C. *sip3Δ* strain is used in panels A to C. β-Galactosidase activities are shown as a percentage of fully induced activity in wild type strain carrying an empty control vector grown in iron-deficient and iron-replete LIM. The error bars represent ± 1 standard deviation for experiments performed in triplicate. (A) The repression of β-galactosidase activities of the p*FET3-lacZ* reporter by overexpression of Izh1p, Izh3p, and Izh4p were absent in the *sip3Δ* mutant strain. (B) The repression of β-galactosidase activities of the p*FET3-lacZ* reporter by overexpression of AdipoR1, AdipoR2 with adiponectin, PAQR3, and PAQR4 were absent in the *sip3Δ* mutant strain. (C) The repression of β-galactosidase activities of the p*FET3-lacZ* reporter by overexpression of mPRα with progesterone, mPRβ, mPRγ, and PAQR11 were absent in the *sip3Δ* mutant strain.





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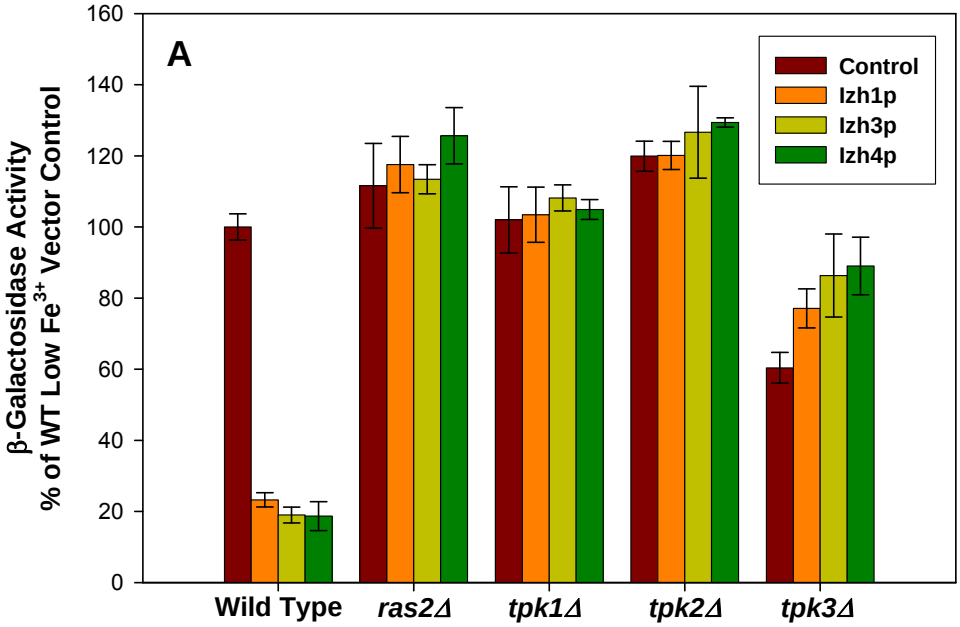
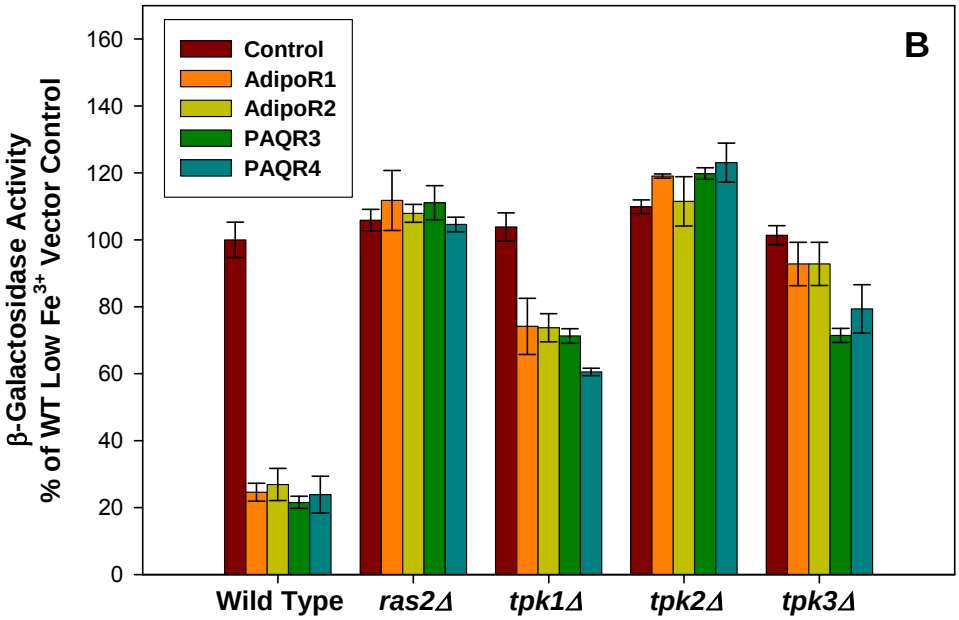
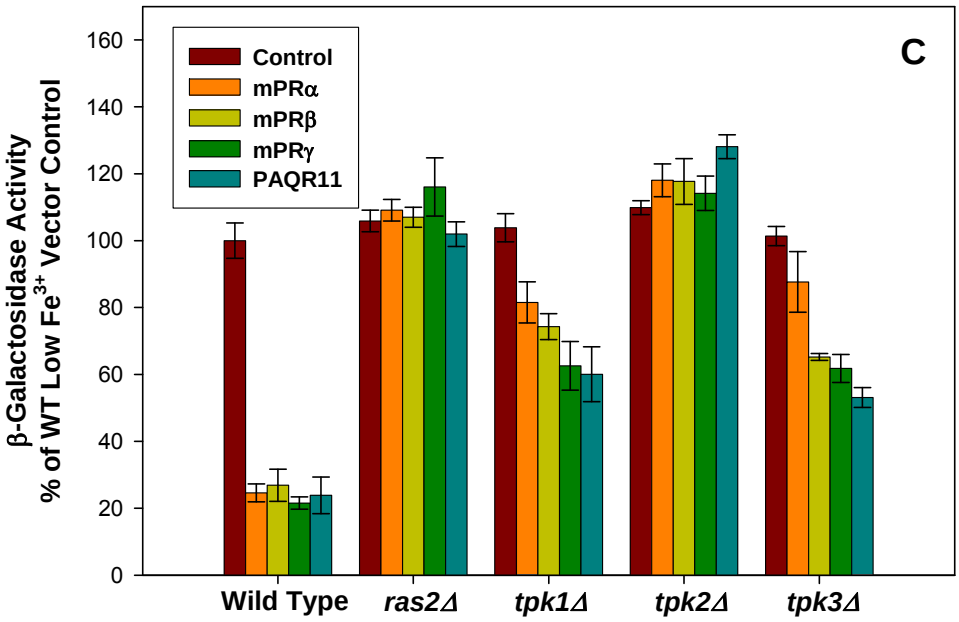


Figure 5-5. The role of Ras2p and PKA in PAQR-dependent repression: BY4742 wild type, *ras2Δ*, *tpk1Δ*, *tpk2Δ*, and *tpk3Δ* strains are used in panels A and B. β-Galactosidase activities are shown as a percentage of fully induced activity in wild type strain carrying an empty control vector grown in iron-deficient and iron-replete LIM. The error bars represent ± 1 standard deviation for experiments performed in triplicate. (A) The repression of β-galactosidase activities of the p*FET3-lacZ* reporter by overexpression of Izh1p, Izh3p, and Izh4p were absent in the *ras2Δ*, *tpk1Δ*, *tpk2Δ*, and *tpk3Δ* mutant strains. (B) The repression of β-galactosidase activities of the p*FET3-lacZ* reporter by overexpression of AdipoR1, AdipoR2 with adiponectin, PAQR3, and PAQR4 were absent in the *ras2Δ*, *tpk1Δ*, *tpk2Δ*, and *tpk3Δ* mutant strains. (C) The repression of β- galactosidase activities of the p*FET3-lacZ* reporter by overexpression of mPRα with progesterone, mPRβ, mPRγ and PAQR11 were absent in the *ras2Δ*, *tpk1Δ*, *tpk2Δ*, and *tpk3Δ* mutant strains.





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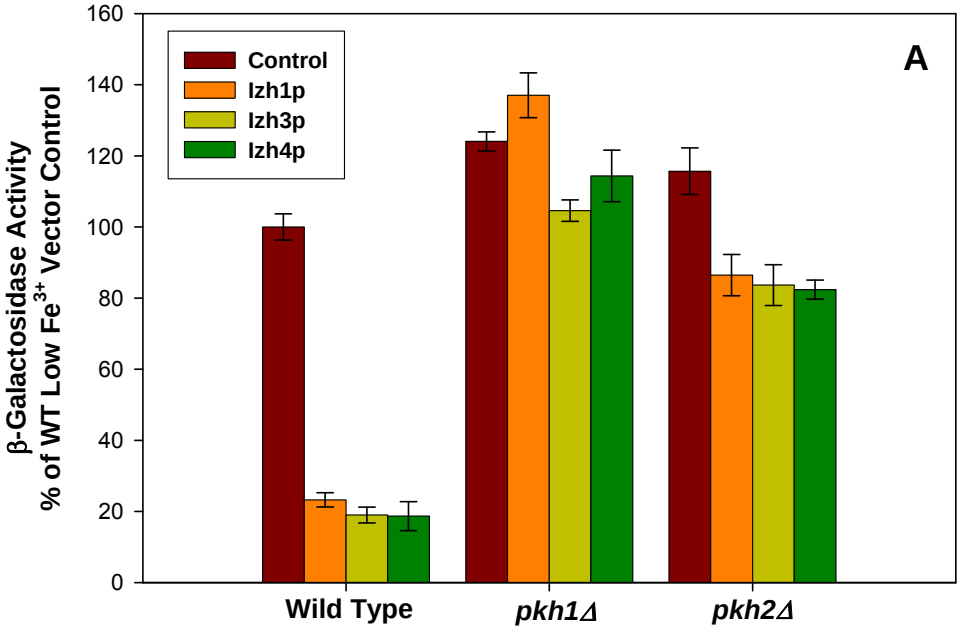
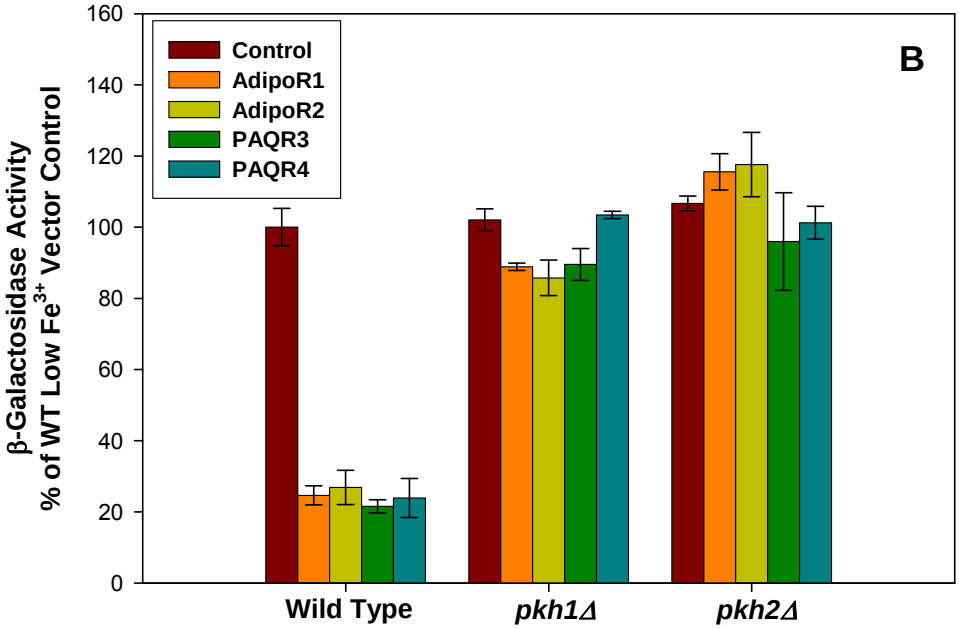


Figure 5-6. PDK is essential for human PAQR-dependent repression: BY4742 wild type, *pdk1Δ*, and *pdk2Δ* strains are used in panels A to C. β-Galactosidase activities are shown as a percentage of fully induced activity in wild type strain carrying an empty control vector grown in iron-deficient and iron-replete LIM. The error bars represent ± 1 standard deviation for experiments performed in triplicate. (A) The repression of β-galactosidase activities of the p*FET3-lacZ* reporter by overexpression of Izh1p, Izh3p, and Izh4p were absent in the *pdk1Δ* and *pdk2Δ* mutant strains. (B) The repression of β-galactosidase activities of the p*FET3-lacZ* reporter by overexpression of AdipoR1, AdipoR2 with adiponectin, PAQR3, and PAQR4 were absent in the *pdk1Δ* and *pdk2Δ* mutant strains. (C) The repression of β-galactosidase activities of the p*FET3-lacZ* reporter by overexpression of mPRα with progesterone, mPRβ, mPRγ and PAQR11 were absent in the *pkh1Δ* and *pkh2Δ* mutant strains.



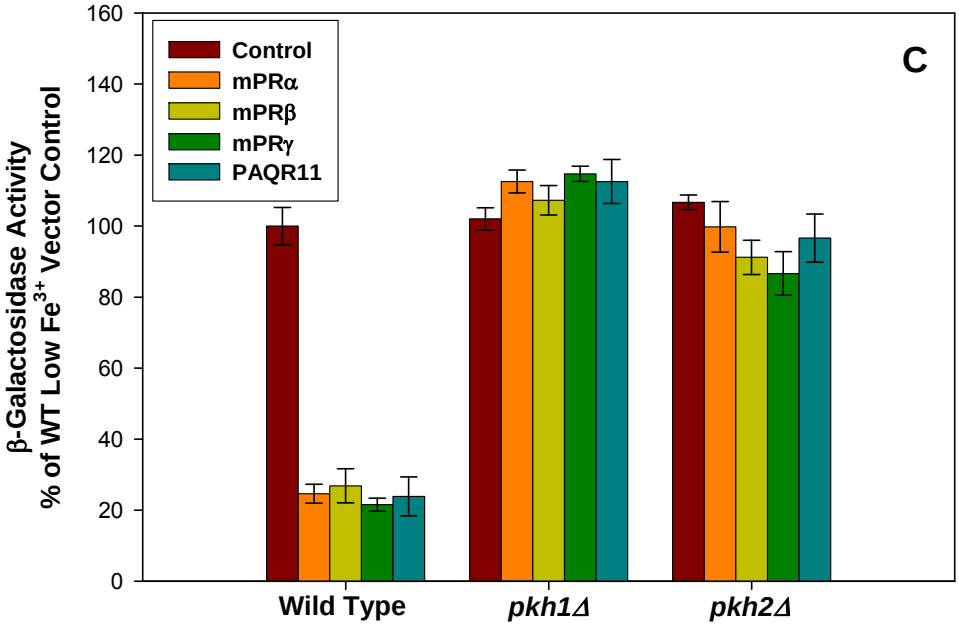


Figure 5-6. Continued

CHAPTER 6
THE EFFECTS OF ADIPONECTIN AND TUMOR NECROSIS FACTOR ON ADIPOR1

Introduction

In the last twenty years, obesity has reached epidemic levels in the United States (Zimmet and Thomas, 2003). Obesity can lead to the problems such as insulin resistance, type 2 diabetes, vascular disorders, and many chronic diseases (Cummings and Schwartz, 2003). Many of these disorders result from altered immune function brought on by an increase of adipose tissue (Channon and Guzik, 2002).

Adipose tissue, originally thought as an energy storage source, has important roles in endocrine and immune systems (Trujillo and Scherer, 2006). This tissue also plays a role in controlling metabolism by secreting a variety of bioactive mediators known as adipocytokines (Rajala and Scherer, 2003). One of the proteins, adiponectin, is found in high levels in adipose tissue, improves insulin sensitivity and decreases inflammation (Scherer et al., 1995). Adiponectin levels are decreased in individuals with insulin resistance and type 2 diabetes, but levels increase after weight loss (Hu and Spiegelman., 1996). Another protein secreted by adipose tissue is the proinflammatory tumor necrosis factor-α (TNF-α), which is structurally related to adiponectin and has increased levels in obesity (Klover, et al., 2003).

Two adiponectin receptors (AdipoR1 and AdipoR2) exhibit binding to adiponectin (Yamauchi, 2003). Both AdipoR1 and AdipoR2 genes encode membrane proteins that belong to a large family known as the Progestin/AdipoQ Receptors (PAQRs) (Tang, 2005). The PAQRs are comprised of three classes, of which AdipoR1 and AdipoR2 are class I proteins. This family of proteins contains at least seven transmembrane domains and similar predicted topology with the cytoplasmic N-terminus and extra-cytoplasmic C-terminus, except for the class II PAQRs

that contain an eighth transmembrane domain (Tang, 2005). Moreover, these proteins contain three conserved motifs found on the cytoplasmic side of the membrane.

We previously demonstrated that overexpression of AdipoR1 in yeast has a measurable phenotype (Kupchak et al., 2007). For instance, AdipoR1 protein overexpression, using a *GAL1* inducible promoter, results in repression in low iron media (LIM) of the gene encoding Fet3p, a component of the high affinity iron-uptake complex. In this study, we show that AdipoR1 overexpression with the addition of adiponectin can repress a p*FET3-lacZ* reporter in low iron media (LIM). Also, TNF-α can partially alleviate repression of FET3 caused by AdipoR1 overexpression in iron-deficient LIM. However, when adiponectin and TNF-α were added simultaneously, repression was not alleviated in LIM with galactose or raffinose. In this study, we also demonstrated--using flow cytometry--that FAM-adiponectin and FITC TNF-α exhibited binding to AdipoR1 on the surface of yeast spheroplasts. Additionally, we showed that unlabeled adiponectin lowered binding of FITC TNF-α; however, FAM-adiponectin binding was only alleviated at high levels of unlabeled TNF-α. Thus, we report that TNF-α can affect and bind to AdipoR1, even though AdipoR1 prefers binding to adiponectin.

Materials and Methods

Yeast Strains and Plasmids

Wild type (BY4742) and *izh1Δizh2Δizh3Δizh4Δ* mutant strains were obtained from Euroscarf and their genotypes are listed in supplementary table 1. The p*FET3-lacZ* plasmid (Gift of D. Eide) has the promoter region of the *FET3* gene fused to the *lacZ* open reading frame and is *URA3* selectable. AdipoR1 and PAQR11 genes were cloned from genomic DNA into pRS316-*GAL1* (*LEU2*) via homologous recombination as previously described (Lyons, et al., 2004).

Primers used to clone AdipoR1 and PAQR11 are listed in supplementary table 2. All clones were sequenced to ensure that no errors occurred in the PCR.

β -Galactosidase Assays

β -Galactosidase and ferroxidase assays were performed as described in chapter 2 except for the addition of the following compounds to LIM. Adiponectin (Biovendor Laboratory Medicine, Inc. #RD172029100), tumor necrosis factor-α (Chemicon International #GF023) and tumor necrosis factor-β (Fitzgerald Industries International #RDI-301BH) were added to the cultures at varied concentrations.

Spheroplast Formation

Spheroplasts for the *izh1Δizh2Δizh3Δizh4Δ* mutant strain were prepared as reported by Holcombe et al (1987), with modifications. A single colony was picked and placed in synthetic dextrose (SD) medium and grown to an OD_{600} ~ 5.0. The culture was reinoculated into synthetic galactose (SGal) media at an OD_{600} of 0.2 and grown to OD_{600} between 1.5 and 2.0. Cells were centrifuged at 4,000 rpm, and washed with 20 mL 0.1 M Tris-Cl, pH 9.4, 10 mM dithiothreitol, 50 mM β-mercaptoethanol, and incubated for 30 minutes with gentle shaking at 30ºC. After centrifugation, the pellet was washed in spheroplasting buffer (1.2 M sorbitol, 10 mM potassium phosphate, 0.5x YPD), centrifuged and resuspended into spheroplasting buffer with 12,500 units of lyticase at an OD_{600} of 25. Culture was incubated at 30ºC with gentle shaking for 45 minutes, and washed twice with 1x PBS. The spheroplasts were recovered by centrifuging at 500 rpm for 10 minutes, then taking off the supernantant. The supernatant was diluted with 1x PBS to an OD_{600} of 0.05.

Addition of Fluorophores

Mono-5-(and-6)-carboxyfluoresein (FAM)-Adiponectin (Phoenix Pharmauceticals, Inc. #FG-ADI-01-A) at varied concentrations was added directly to the spheroplast suspension on

ice. The TNF-α could not be added directly; therefore a commercially available kit (Human TNF-α Biotin Conjugate Kit, R&D Systems #NFTA0) designed to detect binding of TNF-α to humans was successfully modified to detect binding to yeast spheroplasts. 10 μL of biotinylated rhTNF-α at varied concentrations was added to the spheroplast suspension and incubated for 60 minutes at 4ºC. To the spheroplast suspension, 10 μL of avadin-fluorescein isothiocyanate (FITC) reagent was added and then incubated for 30 minutes at 4ºC in the dark. Spheroplasts were washed twice with 1x RDF1 buffer and resuspended in 0.2 mL of 1x RDF1 buffer. For competitive binding assays, nonlabeled adiponectin, TNF-α, or TNF-β was added at varied concentrations to the spheroplast suspension.

Flow Cytometry

Flow cytometry analysis was run on a FACS Vantage SE Turbosort (BD Biosciences, San Jose, CA); the instrument utilizes Cell Quest software from BD Biosciences to collect and analyze data. Data was shown as "% of Ligand Binding to Spheroplasts" and signifies percent of bound ligand to spheroplasts with AdipoR1 or PAQR11 overexpressed, minus percent of bound ligand to control cells. Percent of ligand is a measurement of number of cells bound to the fluorophore, divided by 50,000 cells counted, multiplied by 100. In order to measure the number of cells bound to fluorophore, unlabed cells were initially measured and visualized in a scattergraph, which has two regions. The unlabeled cells were placed in region R2. As the fluorophore was added, binding to the specific ligand by the cells was shifted to region R1. R1 then would be divided by total number of cells counted and turned into a percent to give ligand bound to control, AdipoR1, or PAQR11.

Results

The surface location of an adiponectin receptor was established in previous studies, as localized to the plasma membrane in 3T3-L1 adipocytes (Bogan and Lodish., 1999).

Adiponectin has been shown to bind to AdipoR1 and activate AMP-kinase (AMPK) in mouse skeletal muscle (Yamauchi, 2003; Yamauchi, 2002). In yeast, AdipoR1 overexpression signals through AMPK to repress *FET3* expression in galactose containing iron-deficient LIM (Kupchak et al., 2007). Therefore, we examined adiponectin's ability to elicit an effect on *FET3*. We also examined the effect of TNF-α on *FET3* during AdipoR1 overexpression, because we found sequence homology performing BLAST searches between adiponectin and TNF-α. To see if adiponectin and TNF-α binds to AdipoR1 in yeast, we made spheroplasts that gave us access to the plasma membrane.

Effect of AdipoR1 on *FET3* Expression

The p*FET3-lacZ* reporter responds to the amount of iron added to LIM. It is fully induced during iron-deficiency (1 μM) and repressed during iron-replete (1 mM) conditions. The induction during iron-deficiency is repressed by AdipoR1 gene overexpression (Figure 6-1A). We also demonstrated that repression of p*FET3-lacZ* by AdipoR1 overexpression can be incrementally diminished by decreasing the galactose concentration, which is the inducer of PAQR expression (Figure 6-1B).

Adiponectin Binds to AdipoR1 on Yeast Spheroplasts

p*FET3-lacZ* expression is restored at low concentrations (0.05%) of galactose. However, activity of p*FET3-lacZ* starts to decline at 0.1 pM adiponectin and is repressed at 10 pM adiponectin (Figure 6-2A). To ensure that adiponectin binds to AdipoR1 overexpressed on spheroplasts, we performed flow cytometry studies using FAM-adiponectin. Binding to AdipoR1 was exhibited at 1 pM, and demonstrated saturation at 20 pM (Figure 6-2B).

TNF-α binds to AdipoR1 on Spheroplasts

We examined whether TNF-α exhibited an effect on p*FET3-lacZ* activity when AdipoR1 is overexpressed in galactose containing LIM. Repression of p*FET3-lacZ* caused by TNF-α is

partially relieved at 1 nM and exhibited its maximal effect at 50 nM (Figure 6-3A). TNF-β was also examined, but showed no response (Figure 6-3A). Binding of TNF-α to AdipoR1 overexpressed on spheroplasts shows binding as low as 1nM and saturates the receptor at 40 nM (Figure 6-3B).

Adiponectin, rather than TNF-α, prefers binding to AdipoR1

To examine whether AdipoR1 preferentially binds adiponectin or TNF-α, we performed competitive studies where the addition of adiponectin and TNF-α were measured their effect using β-Galactosidase and flow cytometry binding assays. p*FET3-lacZ* repression in raffinose containing LIM by AdipoR1 overexpression was repressed by 100 pM adiponectin. This repression was not effected by the addition of increasing concentrations of TNF-α (Figure 6-4A). For flow cytometry studies, binding of the fluorophore, FAM-adiponectin to AdipoR1 on spheroplasts was affected only by the addition of 50 nM of TNF-α (Figure 6-4B). The FITC-TNF-α showed less AdipoR1 binding when increasing levels of adiponectin were added (Figure 6-4C).

Discussion

Adiponectin receptor 1 is expressed in many tissues, but is highly enriched in skeletal muscle. AdipoR1 is a 375 amino acid protein (42.4 kDa) consisting of seven transmembrane domains with an intracellular N-terminus (Yamauchi, 2003). AdipoR1 belongs to a family known as the class I PAQRs (Lyons et al., 2004). The PAQRs are conserved from yeast to humans (Klover et al., 2003). The yeast homologue, Izh2p, acts similarly to AdipoR1, regulating lipid metabolism through AMPK activation (Yamauchi, 2003) (Karpichev et al., 2002).

Effect of AdipoR1 on *Fet3* expression

In previous studies, we demonstrated that Izh1p, Izh2p, Izh3p and Izh4p activities could be monitored by using β-galactosidase assays, and that overexpression of any of the Izh proteins

negatively repress *FET3*. This effect was not due to a generalized growth defect, expression of any random protein, or a global defect in transcription, translation, or β-galactosidase activity (Lyons et al., 2004).

Since yeast does not encode an adiponectin receptor, we overexpressed the AdipoR1 gene to study the protein. Similarly to Izh2p, AdipoR1 represses *FET3* in the absence of its activating ligand – adiponectin (Kupchak et al., 2007). It is possible that AdipoR1 in yeast has an unidentified endogenous ligand, and this hypothetical molecule may be present in a high concentration to activate AdipoR1 when it is overexpressed. It is also possible that AdipoR1, like many signal proteins, has an intrinsic basal signaling capability that is amplified by overexpression, making the presence of an activating ligand unnecessary. In the latter case, overexpression would function equivalently to activation. To test this model, we performed the experiment with raffinose, instead of galactose containing LIM. We postulate raffinose decreased the expression of the AdipoR1 protein, thereby inhibiting activation and limiting the signaling capability to repress *FET3*.

Adiponectin binding to AdipoR1 on spheroplasts

Adiponectin is a protein secreted by adipocytes and found at high concentrations (μg/mL) in healthy individuals. Unlike other adipocytokines, whose levels increase with fat mass, adiponectin concentration decreases with obesity (Arita et al., 1999). Adiponectin levels also drop in patients with type 2 diabetes (Hotta et al., 2000), people with reduced vascular function, and people with increased cornary artery disease (Goldstein and Scalia, 2004).

Adiponectin structurally is a heteorotrimer (244 amino acids, 24 kDa), consisting of a N-terminal collagen-like sequence and a C-terminal globular region (Fruebis et al., 2001) and belongs to the complement C1q family (Crouch, 1994; McCormack et al., 1997; Wong et al., 2004).

Unlike Izh2p, AdipoR1 has a commercially available ligand-adiponectin. Therefore, we tested ligand-receptor activation. Increasing concentrations of adiponectin added to AdipoR1 overexpressing cells in raffinose containing LIM caused an incremental repression of *FET3*. Even though AdipoR1 protein expression was lowered, *FET3* repression may occur due to the ligand binding and activating the signaling pathway.

We proceeded to test binding of adiponectin to AdipoR1 on spheroplasts. Spheroplasts were used because AdipoR1 was localized to the plasma membrane (Yamauchi, 2003) and because yeast contain a cell wall, which must be removed to allow efficient binding. To examine binding, fluorescently labeled adipoectin was added and exhibited a high level of binding to cells expressing AdipoR1. PAQR11, a class III PAQR homologue, also was investigated due to its sequence relation to AdipoR1 (Klover et al., 2003) and it's supposed inability to bind to adiponectin. As expected, adiponectin only exhibited binding to AdipoR1 at high concentrations and this could be attributed to nonspecific binding to the spheroplasts. This result indicates that yeast spheroplasts are a viable model to examine human membrane receptor binding.

TNF-α binding to AdipoR1 on spheroplasts

TNF-α is a 17 kDa soluble protein secreted by adipocytes. Its level is increased during diabetes (Wong et al., 2006). TNF-α was examined because it showed homology to adiponectin and features common to all C1q proteins (Scherer et al., 1995). Also, other ligands have been shown to bind to AdipoR1. Osmotin, a receptor for Izh2p (Narasimhan et al., 2005), has no sequence homology with adiponectin; however, it shows structural similarity to adiponectin and can bind and activate AdipoR1 (Narasimhan et al., 2005). TNF-α was tested to examine an effect on *FET3* repression. TNF-α exhibited an increase of *FET3* induction, with an increase in concentration of TNF-α. We postulate TNF-α may act on AMPK via AdipoR1 and cause localization to the nucleus, and repress the negative regulators of *FET3*, Nrg1p and Nrg2p,

thereby inducing *FET3*. In the literature, TNF-α has been shown to reduce expression of adiponectin from 3T3-L1 adipocytes (Fasshauer et al., 2002); thereby AdipoR1 overexpression may be unable to activate signaling and repress *FET3*. TNF-β was also studied because it exhibited structurally similarity to TNF-α, as a "pear"/"cone" shaped trimer (Eck and Sprang, 1989; Eck et al., 1992). However, unlike TNF-α, TNF-β was unable to cause an effect on *FET3*. This may be a result from TNF-β only sharing 30% identity with TNF-α (Pennica et al., 1984).

Since, TNF-α alleviated *FET3* repression caused by AdipoR1, we examined TNF-α binding to AdipoR1 on spheroplasts. TNF-α exhibited binding to AdipoR1, but at a 1,000 times greater concentration than adiponectin. Even though TNF-α showed a lower binding percentage at a greater concentration, this was expected since TNF-α is not AdipoR1's native ligand. However, this is a significant result because it showed TNF-α can bind to AdipoR1.

Adiponectin, rather than TNF-α, prefers binding to AdipoR1

Adiponectin and TNF-α are two cytokines secreted by adipocytes; however, they have a reciprocal relationship in humans (Maeda et al., 2002). Previous studies have shown normal concentrations of adiponectin suppress TNF-α in macrophages (Ouchi et al., 2000). Therefore, we investigated adiponectin and TNF-α simultaneous effect on *FET3* when AdipoR1 was overexpressed. Results showed adiponectin at lower concentrations than TNF-α still repressed *FET3*. To verify these results, we also investigated the effect of an unlabeled ligand on a fluorinated ligand's ability to bind to AdipoR1. In both situations, AdipoR1 preferentially bound to adiponectin. This effect on *FET3* and on binding was not a surprise since adiponectin is AdipoR1's native ligand. Although this study exhibited binding of adiponectin and TNF-α to the receptor, we were unable to calculate the binding affinity of these two ligands to the receptor because protein receptor concentration could not be obtained.

Summation

Our findings support three main conclusions. First, AdipoR1 activates a signal transduction pathway that represses *FET3* expression, while the addition of TNF-α alleviates *FET3* repression. Second, AdipoR1, overexpressed on the spheroplasts' surface, binds adiponectin and TNF- α. Third, AdipoR1 preferentially binds adiponectin over TNF-α.

Future experiments will focus on two main questions. First, can TNF-α bind to AdipoR1 in mammalian cells, or does binding to TNF-α only occur in yeast? Second, what is the effect of adiponectin and TNF-α on AMPK activity when AdipoR1 is overexpressed? We predict an increase in AMPK activity with the addition of adiponectin and a decrease in activity when TNF-α is added. If there is an effect on AMPK by TNF-α, then we can state that TNF-α not only binds to AdipoR1, but can also alleviate *FET3* repression through AMPK deactivation.

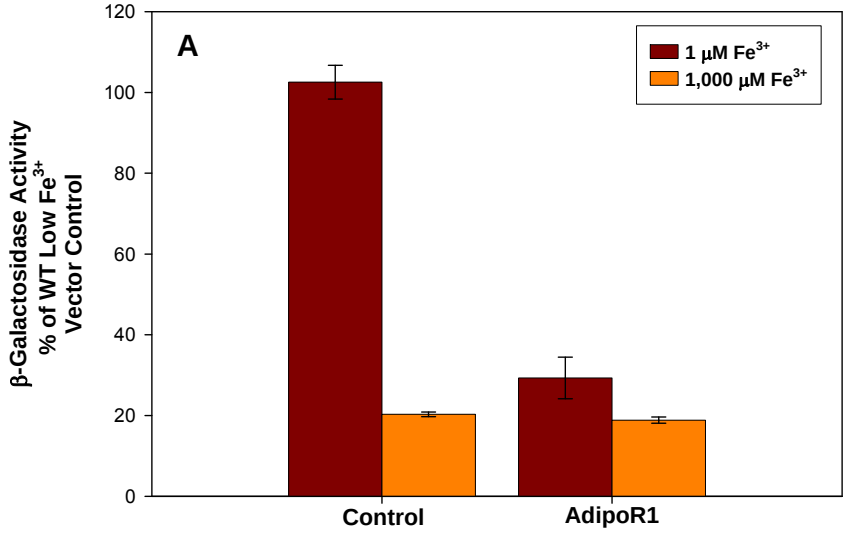


Figure 6-1. AdipoR1 represses *FET3* transcription: BY4742 wild type strain is used in panels A and B. β-Galactosidase activities are shown as a percentage of fully induced β-galactosidase activity in wild type strain carrying an empty control vector grown in iron-deficient LIM. The error bars represent ± 1 standard deviation for experiments performed in triplicate. (A) β-Galactosidase activities of the p*FET3-lacZ* reporter are induced in LIM in the wild type strain carrying the AdipoR1 overexpressor. (B) The AdipoR1 effect on p*FET3-lacZ* decreases as the percent of galactose in iron-deficient LIM is decreased. Activities for each galactose concentration are normalized to percent of activity in a strain carrying an empty control vector grown at the same concentration.

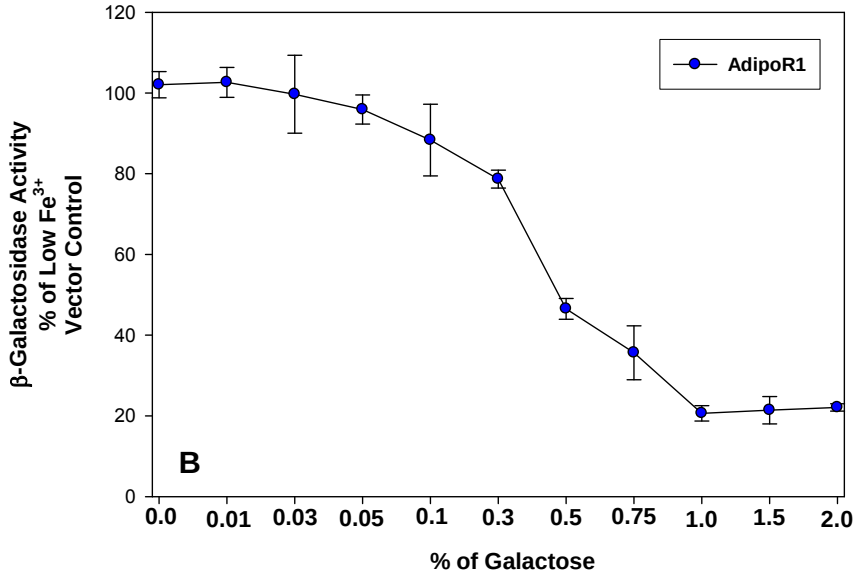


Figure 6-1. Continued.

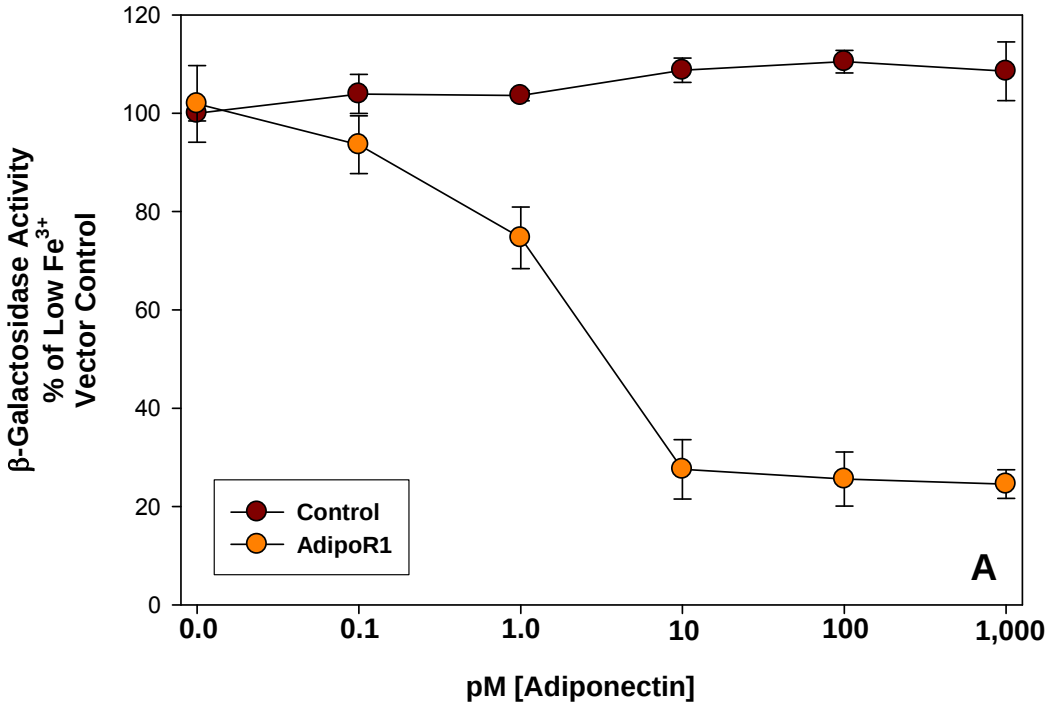


Figure 6-2. Adiponectin binds to AdipoR1 on spheroplasts: For panel A, β-galactosidase activities are shown as a percentage of fully induced β-galactosidase activity in wild type strain carrying an empty control vector grown in iron-deficient LIM. For panel B, binding is shown as percentage of FAM-adiponectin binding to spheroplasts in the *izh1Δ izh2Δ izh3Δ izh4Δ* strain carrying an AdipoR1 or PAQR11 overexpressor, minus the percentage of FAM-adiponectin binding to spheroplasts in the *izh1Δ izh2Δ izh3Δ izh4Δ* strain carrying the empty control vector in SGal. For both panels, the error bars represent ± 1 standard deviation for experiments performed in triplicate. (A) AdipoR1 overexpression in 1.98% raffinose/0.02% galactose exhibits no effect on the p*FET3-lacZ* reporter unless adiponectin is added, while adiponectin alone has no effect. For cells grown in 1.98% raffinose/0.02% galactose, activities are normalized to percent of activity in a wild type strain carrying the empty control vector grown in iron-deficient LIM with the same concentration of raffinose/galactose. (B) FAM-adiponectin in SGal exhibits binding to AdipoR1 overexpressed spheroplasts, while PAQR11 overexpressed spheroplasts demonstrates a low percentage of binding to FAM-adiponectin.

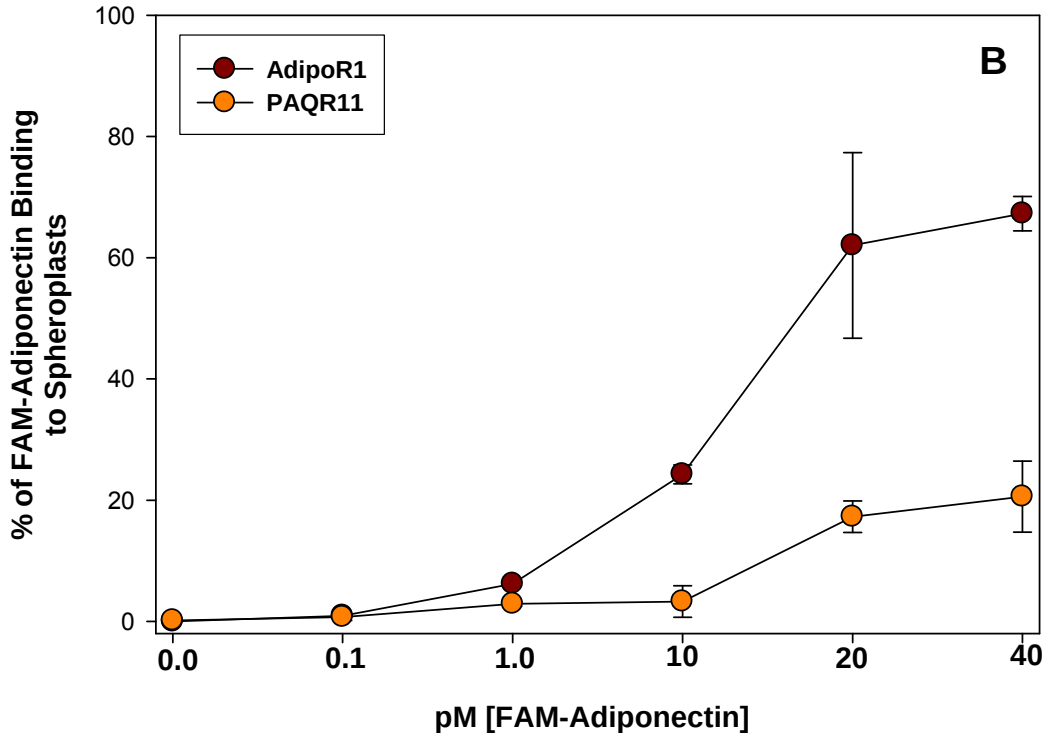


Figure 6-2 continued.

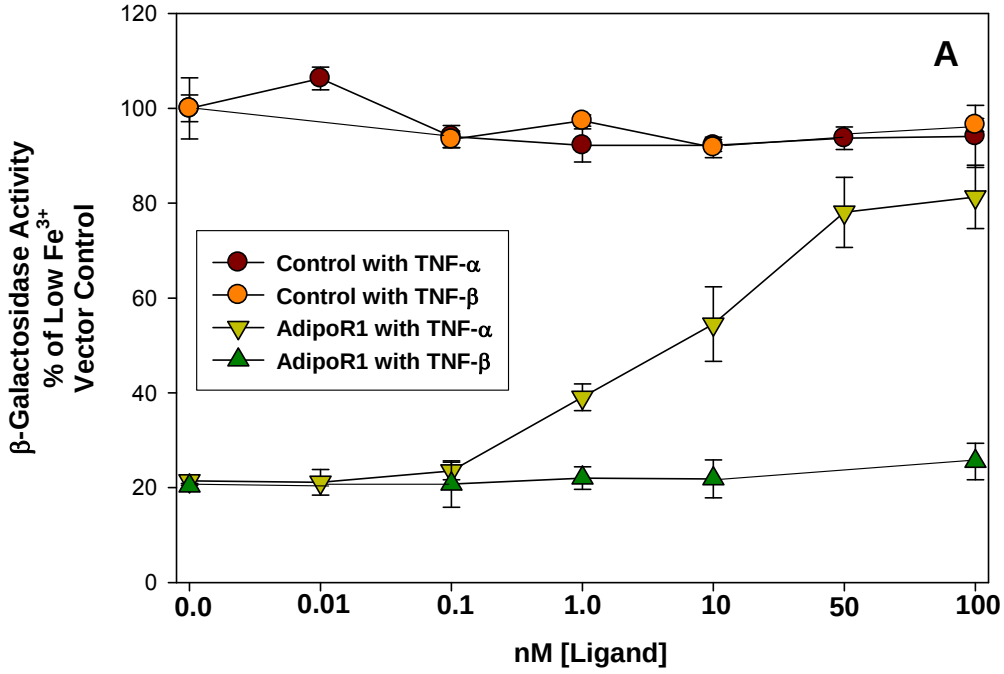


Figure 6-3. TNF-α binds to AdipoR1 on spheroplasts: For panel A, β-galactosidase activities are shown as a percentage of fully induced β-galactosidase activity in wild type strain carrying an empty control vector grown in iron-deficient LIM. For panel B, binding is shown as percentage of FITC TNF-α binding to spheroplasts in the *izh1Δ izh2Δ izh3Δ izh4Δ* strain carrying an AdipoR1 or PAQR11 overexpressor, minus the percentage of FITC TNF-α binding to spheroplasts in the *izh1Δ izh2Δ izh3Δ izh4Δ* strain carrying the empty control vector in SGal. For both panels, the error bars represent ± 1 standard deviation for experiments performed in triplicate. (A) β-Galactosidase activities of the p*FET3-lacZ* reporter are partially induced in the wild type strain carrying the AdipoR1 overexpressor when TNF-α is added, but is not induced by the addition of TNF-β. The empty control vector in the wild type strain was induced when TNF-α or TNF-β was added. (B) FITC TNF-α in SGal exhibits binding to AdipoR1 overexpressed in spheroplasts, while PAQR11 overexpressed in spheroplasts shows a low percentage of binding to adiponectin.

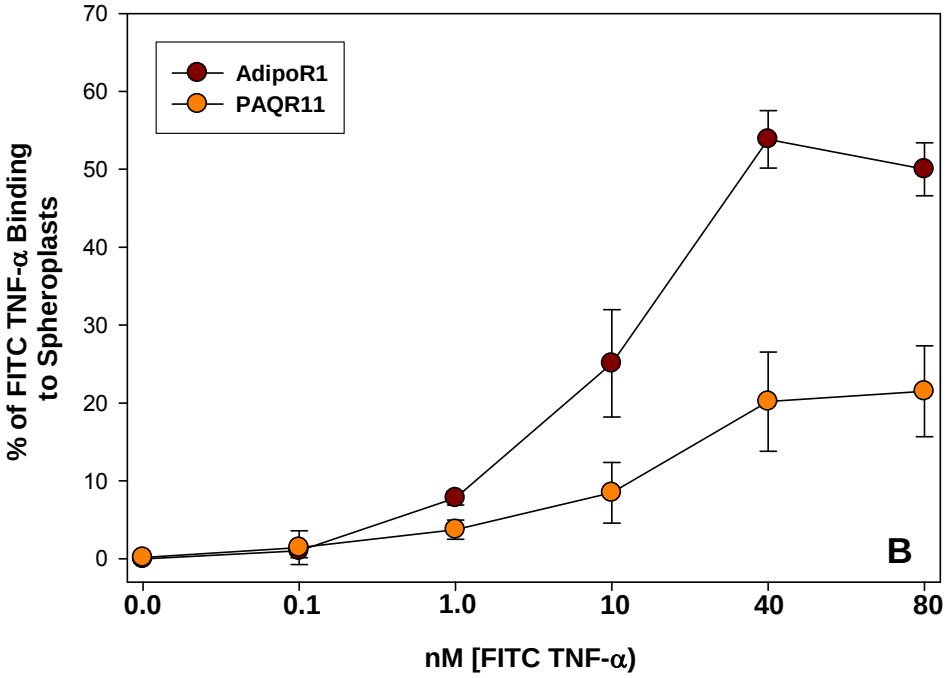


Figure 6-3. Continued.

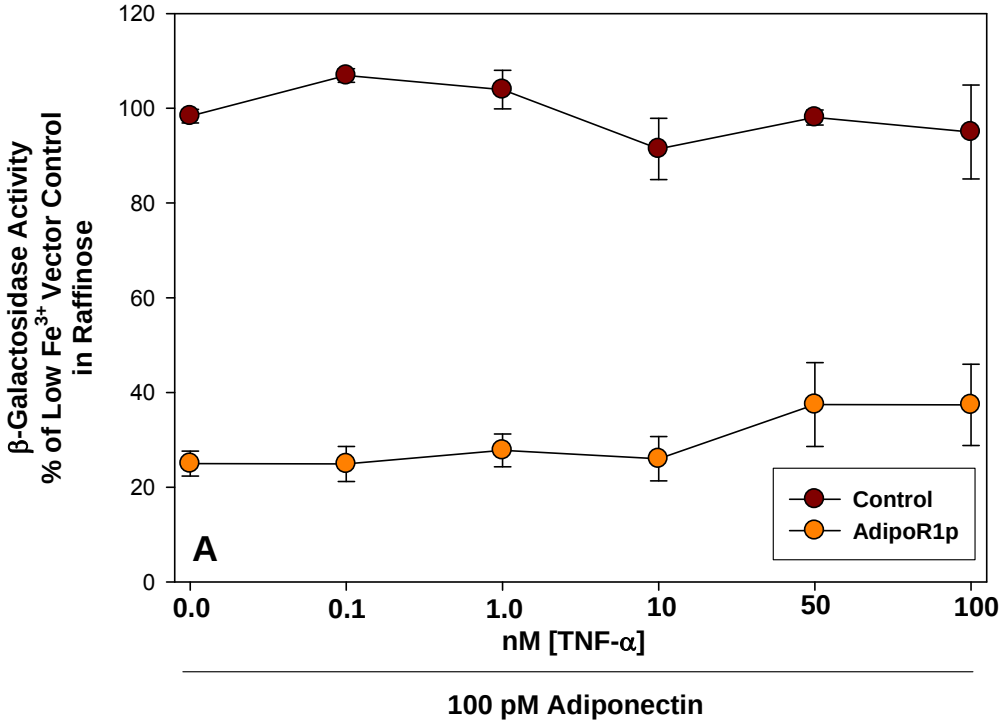
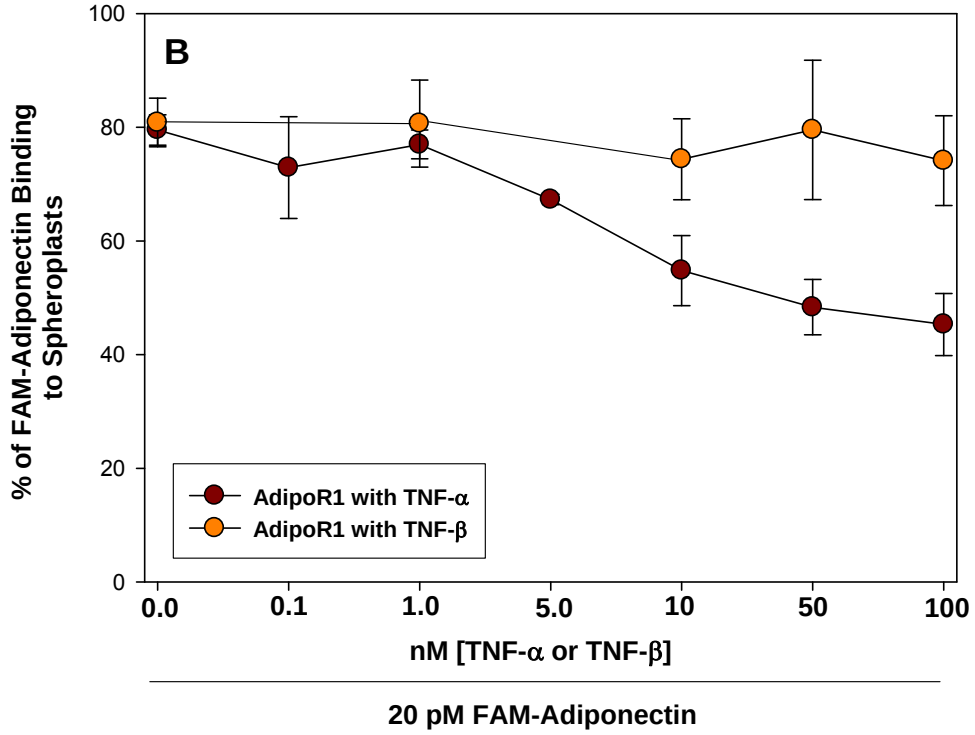


Figure 6-4. Competition of binding to AdipoR1 between adiponectin and TNF-α: For panel A, β-galactosidase activities are shown as a percentage of fully induced β-galactosidase activity in wild type strain carrying an empty control vector grown in iron-deficient LIM. For panels B and C, binding is shown as percentage of FAM-adiponectin or FITC TNF-α binding to spheroplasts in the *izh1izh2Δizh3Δizh4Δ* strain carrying the AdipoR1 overexpressor, minus the percentage of FAM-adiponectin or FITC TNF-α binding to spheroplasts in the *izh1Δizh2Δizh3Δizh4Δ* strain carrying the empty control vector in SGal. For panel A to C, the error bars represent ± 1 standard deviation for experiments performed in triplicate. (A) AdipoR1 overexpressor in 1.98% raffinose/0.02% galactose shows decreased activity of β-galactosidase when adiponectin is added, but the addition of TNF-α did not further affect β-galactosidase activity. For cells grown 1.98% raffinose/0.02% galactose, activities are normalized to percent of activity in a wild type strain grown in iron-deficient LIM with the same concentration of raffinose/galactose. (B) FAM-adiponectin exhibits binding to AdipoR1 overexpressed in spheroplasts, and are effected by the addition of unlabeled TNF-α at high concentrations, whereas the addition of TNF-β did not affect FAM-adiponectin binding to AdipoR1 overexpressed in spheroplasts. (C) FITC TNF-α exhibits binding to AdipoR1 overexpressed in spheroplasts, but binding is lowered with the addition of unlabeled adiponectin.



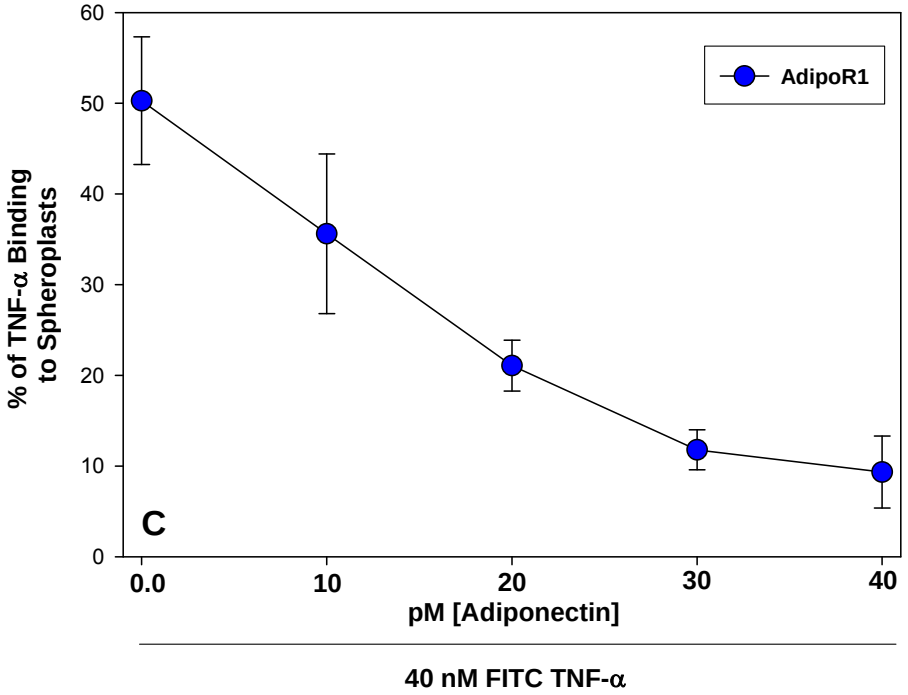


Figure 6-4. Continued.

APPENDIX A
YEAST STRAINS UTILIZED THOUGHOUT THIS STUDY

Strain	Mutation	Source	Genotype
BY4742	Wild Type	Euroscarf	MATα*; his3Δ1; leu2Δ0; lys2Δ0; ura2Δ0*
W303-1A	Wild Type	Dr. Howard Riezman	MATα; *his3Δ1; leu2Δ0; ura2Δ0*
MCY5326	Wild Type	Dr. Marion Carlson	MATα*; his3Δ1; leu2Δ0; ura2Δ0*
YDR490w	*pkh1Δ*	Euroscarf	MATα*; his3Δ1; leu2Δ0; lys2Δ0; ura2Δ0 pkh1Δ::kanMX4*
YOL100c	*pkh2Δ*	Euroscarf	MATα*; his3Δ1; leu2Δ0; lys2Δ0; ura2Δ0 pkh2Δ::kanMX4*
YNL098c	*ras2Δ*	Euroscarf	MATα*; his3Δ1; leu2Δ0; lys2Δ0; ura2Δ0 ras2Δ::kanMX4*
YJL164c	*tpk1Δ*	Euroscarf	MATα*; his3Δ1; leu2Δ0; lys2Δ0; ura2Δ0 tpk1Δ::kanMX4*
YPL203w	*tpk2Δ*	Euroscarf	MATα*; his3Δ1; leu2Δ0; lys2Δ0; ura2Δ0 tpk2Δ::kanMX4*
YKL166c	*tpk3Δ*	Euroscarf	MATα*; his3Δ1; leu2Δ0; lys2Δ0; ura2Δ0 tpk3Δ::kanMX4*
YMR037c	*msn2Δ*	Euroscarf	MATα*; his3Δ1; leu2Δ0; lys2Δ0; ura2Δ0 msn2Δ::kanMX4*
YKL062w	*msn4Δ*	Euroscarf	MATα*; his3Δ1; leu2Δ0; lys2Δ0; ura2Δ0 msn4Δ::kanMX4*
YDR477w	*snf1Δ*	Euroscarf	MATα*; his3Δ1; leu2Δ0; lys2Δ0; ura2Δ0 snf1Δ::kanMX4*
YGL115w	*snf4Δ*	Euroscarf	MATα*; his3Δ1; leu2Δ0; lys2Δ0; ura2Δ0 snf4Δ::kanMX4*
YER027c	*gal83Δ*	Euroscarf	MATα*; his3Δ1; leu2Δ0; lys2Δ0; ura2Δ0 gal83Δ::kanMX4*
YDR422c	*sip1Δ*	Euroscarf	MATα*; his3Δ1; leu2Δ0; lys2Δ0; ura2Δ0 sip1Δ::kanMX4*
YGL208w	*sip2Δ*	Euroscarf	MATα*; his3Δ1; leu2Δ0; lys2Δ0; ura2Δ0 sip2Δ::kanMX4*
YKL048c	*elm1Δ*	Euroscarf	MATα*; his3Δ1; leu2Δ0; lys2Δ0; ura2Δ0 elm1Δ::kanMX4*
YGL179c	*tos3Δ*	Euroscarf	MATα*; his3Δ1; leu2Δ0; lys2Δ0; ura2Δ0 tos3Δ::kanMX4*
YER129w	*sak1Δ*	Euroscarf	MATα*; his3Δ1; leu2Δ0; lys2Δ0; ura2Δ0 sak1Δ::kanMX4*
YDR043c	*nrg1Δ*	Euroscarf	MATα*; his3Δ1; leu2Δ0; lys2Δ0; ura2Δ0 nrg1Δ::kanMX4*
YBR066c	*nrg2Δ*	Euroscarf	MATα*; his3Δ1; leu2Δ0; lys2Δ0; ura2Δ0 nrg2Δ::kanMX4*

YOR171c	*lcb4Δ*	Euroscarf	MATα*; his3Δ1; leu2Δ0; lys2Δ0; ura2Δ0 lcb4Δ::kanMX4*
YLR260w	*lcb5Δ*	Euroscarf	MATα*; his3Δ1; leu2Δ0; lys2Δ0; ura2Δ0 lcb5Δ::kanMX4*
YDR297w	*sur2Δ*	Euroscarf	MATα*; his3Δ1; leu2Δ0; lys2Δ0; ura2Δ0 sur2Δ::kanMX4*
YMR272c	*scs7Δ*	Euroscarf	MATα*; his3Δ1; leu2Δ0; lys2Δ0; ura2Δ0 scs7Δ::kanMX4*
YPL057c	*sur1Δ*	Euroscarf	MATα*; his3Δ1; leu2Δ0; lys2Δ0; ura2Δ0 sur1Δ::kanMX4*
YBR036c	*csg2Δ*	Euroscarf	MATα*; his3Δ1; leu2Δ0; lys2Δ0; ura2Δ0 csg2Δ::kanMX4*
YDR072c	*ipt1Δ*	Euroscarf	MATα*; his3Δ1; leu2Δ0; lys2Δ0; ura2Δ0 ipt1Δ::kanMX4*
YCR034w	*fen1Δ*	Euroscarf	MATα*; his3Δ1; leu2Δ0; lys2Δ0; ura2Δ0 fen1Δ::kanMX4*
YML052w	*sur7Δ*	Euroscarf	MATα*; his3Δ1; leu2Δ0; lys2Δ0; ura2Δ0 sur7Δ::kanMX4*
MCY5338	*msn2Δmsn4Δ*	Dr. Marion Carlson	MATα*; his3Δ1; leu2Δ0; ura2Δ0 msn2Δ::kanMX6; msn4Δ::natMX4*
MCY5378	*nrg1Δnrg2Δ*	Dr. Marion Carlson	*MATα; his3Δ1; leu2Δ0; ura2Δ0 nrg1Δ::hphMX4; nrg2Δ::his3MX6*
MCY5385	*msn2Δmsn4Δ nrg1Δnrg2Δ*	Dr. Marion Carlson	*MATα; his3Δ1; leu2Δ0; ura2Δ0 nrg1Δ::hphMX4; nrg2Δ::his3MX6*

APPENDIX B
PRIMERS USED FOR CLONING

pRS316-*GAL1-IZH1* – 5'
5' – TAC TTC TTA TTC CTC TAC CGG ATC CCG CTC GAG GTC GAC ATG AGT ATC ACC ACT ACT AG – 3'

pRS316-*GAL1-IZH1* – 3'
5' – TGA GCG CGC GTA ATA CGA CTC ACT ATA GGG CGA ATT GGA GCT CGT AGT AGT TGT AGG CCC A – 3'

pRS316-*GAL1-IZH2* – 5'
5'– TAC TTC TTA TTC CTC TAC CGG ATC CCG CTC GAG GTC GAC ATG TCA ACT TTA TTA GAA AGG –3'

pRS316-*GAL1-IZH2* – 3'
5'– TGA GCG CGC GTA ATA CGA CTC ACT ATA GGG CGA ATT GGA GCT CCA AAT ATC TAG GAG ACA AT –3'

pRS316-*GAL1-IZH3* – 5'
5'– TAC TTC TTA TTC CTC TAC CGG ATC CCG CTC GAG GTC GAC ATG ATG GAC TCG TCA AGC A –3'

pRS316-*GAL1-IZH3* – 3'
5'– TGA GCG CGC GTA ATA CGA CTC ACT ATA GGG CGA ATT GGA GCT CAG GAT GTT GTA AGT AAA GG –3'

pRS316-*GAL1-IZH4* – 5'
5'– TAC TTC TTA TTC CTC TAC CGG ATC CCG CTC GAG GTC GAC ATG GTT TCA TTG ACT ACA ATA –3'

pRS316-*GAL1-IZH4* – 3'
5'– TGA GCG CGC GTA ATA CGA CTC ACT ATA GGG CGA ATT GGA GCT CAC CAG CTG AGT CTA AGA –3'

pRS316-*GAL1*-AdipoR1 – 5'
5'- TAC TTC TTA TTC CTC TAC CGG ATC CCG CTC GAG GTC GAC ATG TCT TCC CAC AAA GGA –3'

pRS316-*GAL1*-AdipoR1 – 3'
5'- TGA GCG CGC GTA ATA CGA CTC ACT ATA GGG CGA ATT GGA GCT CTC AGA GAA GGG TGT CAT CA –3'

pYES260-*GAL1*-AdipoR2 – 5'
5'- GGT GGT GGC GAC CAT CAC GAG AAT CTT TAT TTT CAG GGC GCC ATG GGC ATG TCC CCT CTC TT –3'

pYES260-*GAL1*-AdipoR2 – 3’
5’- ATA TCT GCA GAA TTC CAG CAC ACT GGC GGC CGT TAC TAG ATC TTC ACA GTG CAT CCT CTT CAC TGC –3’

pRS316-*GAL1*-PAQR3 – 5’
5’– TAC TTC TTA TTC CTC TAC CGG ATC CCG CTC GAG GTC GAC ATG CAT CAG AAG CTG CTG CTG AA –3’

pRS316-*GAL1*-PAQR3 – 3’
5’– TGA GCG CGC GTA ATA CGA CTC ACT ATA GGG CGA ATT GGA GCT CTC ACA AAT GTG AAA CAT AGT C –3’

pRS316-*GAL1*-PAQR4 – 5’
5’- TAC TTC TTA TTC CTC TAC CGG ATC CCG CTC GAG GTC GAC ATG GCG TTC CTG GCC GG –3’

pRS316-*GAL1*-PAQR4 – 3’
5’- TGA GCG CGC GTA ATA CGA CTC ACT ATA GGG CGA ATT GGA GCT CTC AGT CCC GGG GAC AGG –3’

pRS316-*GAL1*-mPRγ – 5’
5’ TAC TTC TTA TTC CTC TAC CGG ATC CCG CTC GAG GTC GAC ATG CTG AGC CTG AAG CT –3’

pRS316-*GAL1*-mPRγ – 3’
5’- TGA GCG CGC GTA ATA CGA CTC ACT ATA GGG CGA ATT GGA GCT CTG TTG ACA TCT GGC ATG A –3’

pRS316-*GAL1*-mPRα – 5’
5’- TAC TTC TTA TTC CTC TAC CGG ATC CCG CTC GAG GTC GAC ATG GCC ATG GCC CAG AAA –3’

pRS316-*GAL1*-mPRα – 3’
5’- TGA GCG CGC GTA ATA CGA CTC ACT ATA GGG CGA ATT GGA GCT CTC ACT TGG TCT TCT GAT CAA –3’

pRS316-*GAL1*-mPRβ – 5’
5’- TAC TTC TTA TTC CTC TAC CGG ATC CCG CTC GAG GTC GAC ATG ACG ACC GCC ATC TTG –3’

pRS316-*GAL1*-mPRβ – 3’
5’- TGA GCG CGC GTA ATA CGA CTC ACT ATA GGG CGA ATT GGA GCT CTC AGG AAT CTT TCT TGG TCA –3’

pRS316-*GAL1*-PAQR11 – 5’
5’ – TAC TTC TTA TTC CTC TAC CGG ATC CCG CTC GAG GTC GAC ATG CGG TTC AAG AAT CGA TT – 3’

pRS316-*GAL1*-PAQR11 – 3’
5’ – TGA GCG CGC GTA ATA CGA CTC ACT ATA GGG CGA ATT GGA GCT CTC ATA AAT GCC GCA TAA AGT – 3’

APPENDIX C
MEDIA ABREVIATIONS AND COMPOSITION

YPD = Yeast Peptone Dextrose
- 10.0 g/L yeast extract [BD 2010-09-30]
- 20.0 g/L peptone [BD 2009-08-17]

SD = Synthetic Dextrose
- 1.7g/L yeast nitrogen base without amino acids or ammonium sulfate [Difco 2011-05-31]
- 5.0g/L ammonium sulfate [Fisher A702-3]
- 2% w/v glucose [Acros Organics 207-757-8]
- 0.01% appropriate amino acids [Sigma-Aldrich]

SGal = Synthetic Galactose
- 1.7g/L yeast nitrogen base without amino acids or ammonium sulfate [Difco 2011-05-31]
- 5.0g/L ammonium sulfate [Fisher A702-3]
- 2% w/v galactose [Acros Organics 200-416-4]
- 0.01% appropriate amino acids [Sigma-Aldrich]

LZM = Low Zinc Media (1 L)
- 1.7g/L yeast nitrogen base without amino acids or ammonium sulfate [Difco 2011-05-31]
- 5.0g/L ammonium sulfate [Fisher A702-3]
- 2% w/v galactose [Acros Organics 200-416-4]
- 0.01% appropriate amino acids [Sigma-Aldrich]
- 20 mL 1 M sodium citrate, pH 4.2
- 0.2 mL 10 mM manganese chloride
- 0.2 mL 10 mM iron chloride
- 2 mL 500 mM EDTA, pH 8.0
- Zinc deficiency, or zinc repletion was made by adding 1 μM or 1 mM zinc sulfate
- Nano pure water was added to 1 L

LIM = Low Iron Media (1 L)
- 1.7g/L yeast nitrogen base without amino acids or ammonium sulfate [Difco 2011-05-31]
- 5.0g/L ammonium sulfate [Fisher A702-3]
- 2% w/v galactose [Acros Organics 200-416-4]
- 0.01% appropriate amino acids [Sigma-Aldrich]
- 20 mL 1 M sodium citrate, pH 4.2
- 0.2 mL 10 mM manganese chloride
- 0.2 mL 10 mM zinc sulate
- 2 mL 500 mM EDTA, pH 8.0
- Iron deficiency, or iron repletion was made by adding 1 μM or 1 mM iron chloride
- Nano pure water was added to 1 L

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BIOGRAPHICAL SKETCH

Brian Richard Kupchak was born in Scranton, Pennsylvania on May 12, 1978. He grew up in northeastern Pennsylvania and graduated from Scranton High School in June of 1996. It was during this time his love of science became apparent from taking biology and human anatomy. He earned a B.S. in medical technology (2000) during his four years at the University of Scranton. It was here where he realized his true calling was in chemistry. During this time he also became certified as medical technologist and worked at Moses Taylor Hospital from 2000 to 2002.

In 2002, he graduated from the University of Scranton with an M.A. in biochemistry. There, in the laboratory of Dr. Timothy Foley, he studied the "Effects of Reactive Nitrogen Species on Brain Protein Phosphatases." He further enhanced his education by pursuing his Ph.D. in chemistry, beginning in August 2002, at the University of Florida in the laboratory of Dr. Thomas Lyons. Upon completion he will pursue a post-doctoral fellowship in sports nutrition at the University of Connecticut in laboratory of Dr. Jeffery Volek.