TOR SIGNALLING REGULATES CDK8-DEPENDENT INDUCTION OF THE GAL GENES

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ABSTRACT

The GAL genes in \textit{S. cerevisiae} are highly regulated at the transcriptional level and make an excellent model for the study of eukaryotic transcription. GAL transcription is activated by Gal4 in response to the availability of galactose. Gal4 activity is positively regulated by Cdk8-dependent phosphorylation, by an uncharacterized mechanism that becomes essential for GAL induction in yeast lacking the galactose inducer protein Gal3. Null mutations of \textit{cdk8/ srb10}, or mutation of the Cdk8-dependent phosphorylation site on Gal4 at S699, render \textit{gal3} yeast incapable of growing on galactose as the sole source of carbon. The work presented in this thesis exploited this phenotype in a genetic screen to identify mutants that prevent Cdk8-dependent GAL expression with the goal of discovering regulators of Cdk8. I isolated 16 complementation groups, termed the gal four throttle (gft) mutants. One mutant, \textit{gft1}, was found to represent a recessive allele of \textit{hom3}, which encodes an aspartokinase in the biosynthetic pathway for threonine and methionine. Characterization of \textit{gft1} revealed a defect in Tor signalling; strains defective for \textit{gft1/ hom3} are hyper-sensitive to rapamycin and cause constitutive Gat1 nuclear localization. Furthermore, null mutations of \textit{tor1} or \textit{tco89}, encoding components of the TORC1 complex, also prevent GAL expression in \textit{gal3} yeast. Tetrad analysis revealed that \textit{gft7} is allelic to \textit{tco89}. Further genetic analysis demonstrated that disruption of \textit{cdc55}, encoding a regulatory subunit of the PP2A protein phosphatase downstream of Tor signalling suppresses the effect of \textit{gft1/ hom3}, \textit{gft7/ tco89}, and \textit{tor1} mutations on GAL expression. Additionally, a class of \textit{gft} mutants hyper-sensitive to sublethal concentrations of hygromycin B were identified which led to the discovery that \textit{med2} is likely allelic to \textit{gft13-2}. This group of \textit{gft} mutants may represent a mechanistic explanation for the \textit{gft} phenotype that is separate from Tor signalling. Together these
results provide novel insight into how induction of transcription by a specific inducer can be modulated by global physiological signals.
LAY SUMMARY

A genome contains all of the genetic instructions for the construction of an organism. Transcription is the first step in the expression of information coded by a genome. The regulation of transcription allows for the alternative interpretation of genomic information in a context dependent manner. These contexts are potentially limitless and can also be influenced by any combination of the nutritional environments experienced by the organism. Therefore, in order to achieve an appropriate response through gene expression, transcriptional regulation must be accurate and specific. When transcription becomes inaccurate or dysregulated diseases such as cancer arise. In this work, I use Baker’s yeast, *Saccharomyces cerevisiae*, and the GAL gene regulon they possess as a model to gain insight into the regulatory pathways that work upstream of the transcriptional machinery.
This thesis is original, unpublished work performed by the author, Nicole Hawe. Dr. Ivan Sadowski conceptualized this project along with Dr. John Rohde. All experiments except for the gft mutant screen were performed by Nicole Hawe. The original gft screen was completed by Dr. John Rohde and Dr. Ivan Sadowski.
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Chapter 1: INTRODUCTION

1.1 Overview and Rationale

The overarching goal of the work in this thesis was to elucidate how Cyclin-dependent kinase 8 (Cdk8) activity is regulated, using the GAL genes of the yeast, Saccharomyces cerevisiae. Transcriptional regulation is important to study because many diseases are the result of the improper control of transcription and associated events. The mechanisms of eukaryotic transcription are highly conserved. The yeast GAL genes are a well-defined system, controlled by positive and negative stimuli and are valuable for studying the basic mechanisms involved in transcriptional regulation. To identify pathways involved in regulating Cdk8 activity, a genetic screen for defects in Cdk8-dependent induction of the GAL genes was performed in our lab, resulting in isolation of 16 different mutants, which I have characterized to uncover the details behind this mechanism of transcriptional regulation. In this thesis I detail how analysis of this mutant collection has revealed a novel role for nutrient signalling through the target of rapamycin (TOR) signalling pathway for modulation of Gal4 activity and regulation of the GAL genes.

1.2 Yeast as a Model for Eukaryotic Gene Regulation

The budding yeast, Saccharomyces cerevisiae, has proven to be an invaluable model system for the discovery of many eukaryotic biochemical functions, and has been used for decades. The S288C strain of Saccharomyces cerevisiae was the first complete eukaryotic genome to be sequenced (Goffeau et al., 1996). However, long before its genome was sequenced, yeast had been used as a model organism for studying eukaryotic cell biology. The advantages of this organism are numerous, from ease of genetic manipulation, to quick growth
rate on inexpensive media, and stable existence in haploid or diploid states (Botstein, 2011). The ability of budding yeast to mate and sporulate facilitates the rapid combination of various genetic traits and outcrossing of unwanted mutations (Botstein, 2011). Additionally, since budding yeast are eukaryotes they share a significant portion of their encoded proteins with humans. The transcriptional machinery in yeast is highly conserved, and insights into transcriptional regulation are often relevant to other eukaryotes, humans included (Botstein, 2011). The GAL regulon found in yeast has proven to be a particularly useful model for studying gene regulation in eukaryotes. Our understanding of many cellular processes, including transcription has been greatly enhanced through the use of various yeasts as model organisms.

Yeast are capable of utilizing a wide range of different carbon sources. Glucose of course is preferred, as it can enter directly into the glycolytic pathway, however other sugars can be used but must first be converted to glucose by an assortment of enzymes (Gancedo, 1998). The use of alternative sugars is limited in the presence of glucose due to its ability to negatively regulate the expression of these converting enzymes (Carlson, 1999; Kim, et al., 2013). Glucose can act as a negative regulator in two distinct ways; repression where the transcription of these genes is inhibited in the presence of glucose, or inactivation of the protein product by being modified post-translationally usually leading to degradation (Carlson, 1999; Gancedo, 1998).

1.3 Transcriptional Specificity and Regulation

Most cells within an organism contain identical genetic information, yet they can have vastly different morphologies and functions. This is in large part due to transcriptional regulation leading to differential gene expression. To mount an appropriate response ranging from cell growth to metabolic shifts, certain target segments of the genome must be transcribed at specific times under specific conditions (Emerson et al., 2002). This means that the essence of
transcriptional regulation is specificity. The daunting aspect however, is that transcription involves multiple different components, making its regulation very complex (Emerson et al., 2002). Successful transcription requires the concerted action of many transcription factors (TFs) acting as activators or repressors, the multi-subunit enzyme RNA-Polymerase II (RNAPII), and the general initiation factors (GIFs) (Emerson et al., 2001). Malfunction or loss of specificity leads to dysregulation of these components often resulting in disease. However, in order to uncover the true core of these diseases, a complete understanding of the loss of specificity is required. We are in need of a more complete picture of the factors regulating expression of the genome.

Regulation of the process of transcription occurs at many steps. Transcription itself involves three stages: initiation, elongation and termination (Buratowski et al., 1989, Cox et al., 1998). Each of these stages is regulated, however it is thought that initiation is the most regulated in a gene specific manner (Buratowski et al., 1989; Emerson et al., 2002). Once expressed, mRNA can be further post-transcriptionally regulated by capping, splicing, editing and silencing that can affect the stability, and translation of an mRNA molecule. A huge number of proteins come together to carry out transcription and it is at this initial, most heavily regulated step in gene expression, that dysregulation can have the biggest impact on the cell.

1.4 RNAPII Holoenzyme

In order for RNAPII to initiate transcription it requires additional protein complexes and specific sequences on its template DNA. These additional components include general transcription factors (GTFs) which are a class of 7 conserved protein complexes named TFIIA, B, D, E F, and H (TF for transcription factor, II for RNAPII) (Butler, et al., 2002). These associate with RNAPII forming the preinitiation complex (PIC) which is capable of supporting
basal transcription (Butler, et al., 2002; Cox, et al., 1998). Formation of the PIC occurs at specific DNA sequences called core promoters which help define the transcription start site (TSS) and typically contains additional sequence motifs such as a TATA box, which help direct the binding of GTFs (Buratowski et al., 1989; Cox et al., 1998; Fuda et al., 2009). It is thought that at least 60 b.p. of promoter DNA is occupied in the PIC, where nearly every base pair is in contact with RNAPII and/or a GTF (Kim et al., 2000; Miller and Hahn, 2006). High levels of transcription require the additional binding of one or more transcriptional activators which consist of a sequence-specific DNA binding domain, along with an activation domain (Hanh et al., 2011; Kornberg, 2005). Binding sites for activators are usually close to or upstream of the core promoter and are called upstream activation sequences in yeast (UAS) (Hanh et al., 2011). These binding sites can also be found in enhancer regions which can be much further away from the core promoter in metazoan eukaryotes (Hanh et al., 2011). The specificity of DNA-bound activators lies in their ability to recognize sites within a promoter (Hanh et al., 2011). Although binding of a single transactivator can be sufficient to activate transcription, most promoters contain several UASs, allowing for a synergistic effect on the rate of transcription (Hanh et al., 2011; Tjian et al., 1994).

There are currently two competing views on how the PIC forms. The first view supposes a stepwise construction of the PIC which begins with TFIID binding its DNA target, the TATA box. Once this initial scaffold is assembled, the additional GTFs and RNAPII bind in a stepwise manner to form the full PIC (Tjian et al., 1994; Buratowski et al., 1989). The second view is that of a pre-assembled complex, where TFIID still binds the TATA box first however, RNAPII bound to GTFs exists in a pre-assembled complex that then binds the TFIID scaffold as one unit (Bjorklund et al., 1996; Koleske et al., 1995). The key message regarding these two views is that
they both begin with a pre-formed TFIID-TATA box complex and result in a fully functional PIC stabilized by a vast network of noncovalent intermolecular interactions. Therefore, the main difference between the stepwise, and holoenzyme pathways lies in the order of PIC formation not the identities of the interactions within the PIC.

1.5 CTD

The largest subunit of RNAPII, Rpb1, contains a carboxy terminal domain (CTD) comprised of the repeated (26 times in yeast, 52 humans) conserved heptapeptide sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser (Buratowski, 2009; Giuliana et al., 2014). The CTD is dynamically modified in a variety of ways, while some of these modifications occur in a pattern along the CTD during each transcriptional stage, forming what is known as the “CTD code” (Buratowski, 2009; Eick and Geyer, 2013). Phosphorylation is the most extensively understood modification as it can occur at five of the seven residues making up the CTD motif, Y1, S2, T4, S5, and S7 (Buratowski, 2009; Eick and Geyer, 2013). The importance of this repeated sequence is highlighted by lethality resulting in all species when it is completely removed, however a slightly shortened version is tolerated (Corden, 2013).

In yeast, three predominate kinases have been identified that are capable of phosphorylating the CTD (Giuliana et al., 2014). During initiation of transcription Ser5 is phosphorylated by the kinase Kin28/ Cdk7 in the TFIIH complex (Buratowski, 2009; Giuliana et al., 2014). Ser5 phosphorylation aids in promoter clearance along with recruitment of mRNA capping factors which further help process the nascent transcript (Eick and Geyer, 2013). The second kinase capable of phosphorylating CTD is Srb10/ Cdk8, which is the cyclin-dependent kinase component of the Mediator complex (Kim et al., 1994). Cdk8 is capable of Ser2 and Ser5 phosphorylation in vitro however, in vivo the significance of Cdk8 CTD phosphorylation is
unknown (Giuliana et al., 2014; Kim et al., 1994). The transition to productive elongating transcription is connected with high levels of Ser2 phosphorylation which eventually helps to recruit termination factors (Buratowski, 2009; Giuliana et al., 2014). The kinase responsible for this modification has become a complicated story. Originally, it was thought that the kinase Ctk1 contributes to the bulk of Ser2 phosphorylation, which still holds true (Kim et al., 2000; Liu et al., 2009). Recently though, the kinase Bur1 has been shown to increase the levels of Ser2 phosphorylation further (Liu et al., 2009, Wood et al., 2006). In mammals the kinase Cdk9 which is part of the pTEF-b complex phosphorylates Ser2 in the latter stages of active transcription, both Ctk1 and Bur1 resemble mammalian Cdk9 of pTEF-b (Zhou et al., 2009). It is thought that the Ser5P to Ser2P transition either promotes the association and activity of positive elongation factors or inhibits pathways that cause RNAPII to pause or terminate early during transcription (Buratowski 2009).

1.6 Mediator

The search for a “mediator” protein began when the observation was made that GTFs and RNAPII could support basal levels of transcription in vitro however they were unresponsive to transactivators (Flanagan et al., 1991; Kelleher et al., 1990). Over two decades ago, a group performed a screen to find suppressors of a growth defect resulting from a CTD truncation in order to understand the function of the CTD (Koleske and Young 1994). This screen identified a family of nine genes termed SRBs (suppressors of RNA polymerase B) (Koleske and Young, 1994). This group went on to purify a complex which contained not only the SRBs but also several GTFs (Koleske and Young, 1994). The purification of the “holoenzyme” occurred somewhat by accident with the focus being to immunoprecipitate the SRBs but instead they ended up with a complex containing RNAPII, TFIIB, -F, -H, Gal11, the SRB proteins and
another complex, which turned out to be mediator (Koleske and Young, 1994). This complex, unlike the core RNA Pol II, responded to activators in \textit{in vitro} transcription reactions (Koleske and Young, 1994). Around the same time, a holoenzyme complex was independently identified by the Kornberg group through its association with the mediator complex (Kim et al., 1994).

The mediator complex is now known to interact with transcriptional activators as well as directly contacting RNAPII in the PIC. It is thought to be key in recruiting RNAPII to promoters and stabilizing the preinitiation complex at the core promoter (Kornberg, 2005; Lee et al., 1997). Mediator has also been shown to bind the CTD in its unphosphorylated state (Corden, 2013; Young et al., 1994). Once CTD becomes phosphorylated by Kin28/ Cdk7 during initiation Mediator is unable to bind, and it is this cycle of CTD phosphorylation/ dephosphorylation that is thought to control Mediator and RNAPII interaction (Corden, 2013). Additionally, there is structural information showing several activator binding domains in Mediator subunits supporting that this complex is recruited to promoters in an activator-dependent manner (Harper et al., 2017).

The yeast Mediator is comprised of 25 subunits, all of which have homologs in mammals (Kornberg et al., 2005). The Mediator complex is divided into 3 core modules, the head, middle and tail, along with a fourth transient kinase module (Kornberg et al., 2005; Tsai et al., 2014). The yeast Mediator tail module is comprised of Med2, Med3, Med15/Gal11 and Med16/Sin4 subunits (Jeronimo et al., 2016; Tsai et al., 2014). The evolutionarily conserved, 4-subunit subcomplex termed the kinase or Cdk8-module, is composed of CDK8(Srb10), Cyclin C(Srb11), MED12 and MED13 (Kornberg et al., 2005; Tsai et al., 2013). The presence of the CDK8-module bound to Mediator is an alternative form of the Mediator complex, which is usually found in a free form, lacking the presence of RNAPII (Tsai et al., 2013). Currently the theory in
the field is that the Cdk8-module competes with RNAPII for Mediator binding, therefore when the Cdk8 module is bound it represses transcription (Elmlund et al., 2006; Tsai et al., 2013). The association of Cdk8 with Mediator is dynamic, and it has been shown that up to 30% of the total cellular Cdk8 is found independent of Mediator (Tsai et al., 2013).

1.7 The GAL Genes of Saccharomyces cerevisiae

The study of galactose catabolism in yeast began over 50 years ago with the identification of mutants unable to utilize galactose (Robichon-Szulmajster, 1958). This screen led to the characterization of the genes required for the conversion of galactose into glucose-1-phosphate, which is known as the Leloir pathway (Frey, 1996; Robichon-Szulmajster, 1958). The GAL regulon in Saccharomyces cerevisiae is highly regulated at the transcriptional level, and one of the most widely used models for studying the regulation of transcription (Traven et al., 2006). The regulation of the GAL genes in response to changing environmental conditions is done through activating and repressing activities (Kim et al., 2013; Traven et al., 2006). The structural genes of this family are GAL1, GAL2, GAL10 and GAL7, encoding enzymes of the Leloir pathway, whereas the specific regulatory members are GAL3, GAL4, and GAL80 (Kim et al., 2013; Traven et al., 2006). Galactose induces most of these GAL genes while glucose causes strong repression, even when galactose is present, due to glucose being the favourable carbon source (Frey, 1996).

1.7.1 Gal2

Galactose enters the cell through the action of GAL2, which encodes both a high and low affinity facilitated-diffusion transmembrane transporter (Ramos et al., 1989). Surprisingly, Gal2 is capable of transporting glucose as well as galactose (Ramos et al., 1989). However, in the presence of glucose, the transportation of galactose is competitively inhibited, the effect of which
contributes to repression of the GAL genes (Ramos et al., 1989). Strains that are defective in gal2 often present as being unable to efficiently grow on galactose as the sole carbon source, despite being able to induce Gal4 regulated genes (Ramos et al., 1989). Some of the original laboratory strains of Saccharomyces, including S288C, were found to have defects in GAL induction as a consequence of naturally occurring "weak" gal2 alleles (Mortimer et al., 1986).

1.7.2 Gal80

Gal80 is a negative regulator of Gal4 and is responsible for inhibiting GAL gene induction in the absence of galactose (Figure 1) (Leuther et al., 1992). Gal80 has been shown to interact with the activation domain AR2 of Gal4, preventing it from interacting with its GTF and mediator targets (Leuther et al., 1992). Mutations which inactivate Gal80 cause the GAL genes to be expressed constitutively, whereas dominant GAL80 alleles cause yeast to be incapable of expressing the GAL genes (Douglas et al., 1972). Inhibition by Gal80 is relieved upon induction by galactose (Leuther et al., 1992).

1.7.3 Gal3

Gal3 is critical for rapid induction of the GAL genes (Figure 1) (Nogi, 1986). Cells lacking gal3 still induce GAL transcription in response to galactose, however rather than a significant level of induction occurring after hours, it takes several days in a process known as "long term adaptation" (LTA) (Mundkur et al., 1949). Gal3 is nearly identical in sequence to the galactokinase Gal1, the first enzyme of the Leloir pathway, but unlike Gal1 is unable to phosphorylate galactose to produce galactose-1-phosphate (Bhat et al., 1990). Accordingly, it was found that Gal1 expressed at high levels can functionally replace Gal3 for induction (Bhat et al., 1990). Early on it was thought that Gal3 might produce a metabolite of galactose that acted as an inducer, similar to the inducer allolactose of the E. coli Lac operon. Instead, it was
subsequently shown that Gal3 protein itself, rather than a metabolite, is necessary for induction (Nogi, 1986). It was later shown that a galactose-Gal3 complex acts as the inducer binding directly to Gal80 (Blank et al., 1997; Suzuki-Fujimoto et al., 1996). This is an ATP-dependent process which results in a ternary complex with Gal4 and is capable of activating transcription (Reece and Platt, 1997). A competing model exists that describes an alternative function for Gal3 where it sequesters Gal80 in the cytoplasm in a galactose activated fashion (Jiang et al., 2009, Peng and Hopper, 2002). A decrease in the nuclear to cytoplasmic ratio of Gal80 is seen upon galactose induction supporting the idea of sequestration in the cytoplasm (Peng and Hopper, 2002). Amazingly, even though these two models are decades old it still hasn't been conclusively demonstrated which one is right. It is likely that a combination of mechanisms exists, such that Gal3 is required for initial induction by direct interaction and may also sequester Gal80 into the cytoplasm to maintain induction as long as galactose is present.

1.7.4 Gal4

Expression of the GAL regulon in the presence of galactose is regulated by the transactivator Gal4. In the promoters of the GAL genes there are upstream activating sequences (UAS) which contain the Gal4 binding sites (Lohr, 1995). Differential activation and combinatorial expression of these GAL genes is mediated by different numbers of Gal4 binding sites in the UAS, as well as each site having different affinities for Gal4 (Lohr, 1995). In non-inducing conditions (galactose absent) Gal4 is bound to the UAS but is prevented from activating transcription due to binding of the inhibitor Gal80 (Blank et al., 1997; Lohr, 1995; Rhode et al., 2000). This inhibitor binds the C-terminal 30 amino acids of the Gal4 C-terminal activating domain which presumably prevents its interaction with the general transcription factors (Ma and Ptashne, 1987). Relief of
inhibition by Gal80 requires a functional Gal3 bound to galactose (Figure 1) (Nogi, 1986; Rohde et al., 2000).

Upon induction by galactose, Gal4 becomes phosphorylated at multiple serine residues by cyclin-dependent protein kinases of the RNA Pol II holoenzyme (Hirst et al., 1999). Full induction of Gal4 activity requires phosphorylation at its S699 residue and this phosphorylation is mediated by Cdk8/Cyclin C of the mediator subcomplex of the RNA Pol II holoenzyme (Figure 1A) (Rohde et al., 2000). Although other phosphorylation sites exist on Gal4, S699 is the only one that seems to be required for activation of transcription of the GAL genes (Rohde et al., 2000; Sadowski et al., 1996). Disruption of Cdk8 or mutation of the S699 phosphorylation site on Gal4 limits induction to approximately 10-20% of the fully induced GAL genes (Sadowski et al., 1996). Newly developing insight into this complex regulatory system is constantly unfolding, leading to a better understanding of how an organism’s environment can alter the transcription of certain genes.

1.7.5 Long Term Adaptation (LTA)

The mechanism behind the phenomenon of LTA has remained an enigma for a long time. In the earliest characterized laboratory yeast strains it was seen that some were able to ferment galactose rapidly within hours while others required several days to begin fermenting (Mundkur and Lindgren, 1949, Winge and Roberts, 1948). The differences in the response of these strains to galactose was caused by naturally occurring mutations in the GAL3 gene (Torchia and Hopper, 1986). Hopper and colleagues found that respiratory competence and galactose pathway function were indirectly required for induction of LTA in gal3 yeast however the most important requirement was Gal1 (Bhat and Hopper, 1992). This was supported by the fact that overexpression of GAL1 was able to partially suppress LTA in gal3 cells, as well as the inability
of \textit{gal3 gal1} strains to induce \textit{GAL} transcription at all, likely due to the absence of both inducer proteins (Bhat et al., 1990, Bhat and Hopper, 1992). Although the discovery of these requirements for LTA helped understand this phenotype, the mechanism still remained elusive.

A breakthrough came years later when it was found that Cdk8 and phosphorylation of Gal4 at the S699 residue are required for LTA in \textit{gal3} yeast (Rohde et al., 2000). This may in part be explained by a conformational shift between Gal80 and Gal4 that may occur at a spontaneous slow rate in the absence of Gal3 (Figure 1B) (Rohde et al., 2000). When this occurs in yeast with active Cdk8 then Gal4 can be phosphorylated at S699 and induction occurs (Rhode et al., 2000). Since galactose can bind Gal1, and Gal1 can functionally replace Gal3, the eventual accumulation of Gal1 caused by activation of Gal4 by Cdk8 may produce the long-term adaptation phenotype for induction (Rhode et al., 2000). This might explain the sporadic growth of colonies of W303-1A \textit{gal3} yeast on EB-Gal (Rhode et al., 2000). These colonies may represent cells in which Gal80 has spontaneously changed conformation, allowing Cdk8 to phosphorylate Gal4 resulting in induction (Rhode et al., 2000). Although the discovery that Cdk8 phosphorylation of Gal4 S699 is required for induction of LTA was a huge stride forward in understanding this phenomenon it still remains unknown why only a limited population of \textit{gal3} cells induce in this Cdk8-dependent manner. If a spontaneous shift in Gal80 does occur in these cells, it has yet to be determined what causes these specific cells to be more prone to this effect.

\textbf{1.8 The TOR Signalling Pathway}

The Tor pathway is a highly conserved signalling pathway in eukaryotes that controls many aspects of an organisms’ growth, from coordinating nutritional and mitogenic signals to controlling gene expression and protein biosynthesis. The \textit{TOR} genes were initially identified as mutants that confer resistance to a newly discovered potent antiproliferative microbial compound
rapamycin (Heitman et al., 1991; Koltin et al., 1991). In vivo rapamycin binds with high affinity to the prolyl isomerase FKBP12 to form a drug-protein toxin which binds the highly homologous Tor1 and Tor2 proteins in yeast (Heitman et al., 1991; Koltin et al., 1991). Subsequent studies have now revealed TOR homologs conserved from yeast, flies, and humans (Hall, 1996). It should be noted that S. cerevisiae is unusual in having two TOR genes whereas almost all other eukaryotes, including plants, worms, flies, and mammals, have a single TOR gene (Hall, 1996). Disruption of TOR1 alone in yeast typically has little-to-no effect, whereas, disruption of TOR2 alone causes cells to arrest growth (Kunz et al., 1993). It is thought that TOR2 has two essential functions: one function is redundant with TOR1 and the other function is unique to TOR2 (Hall, 1996; Helliwell et al. 1998). This additional complexity in S. cerevisiae helped the analysis of TOR signalling because it allowed differentiation between two functionally different branches, but the redundancy in the functions of the two TORs has made deciphering these pathways more difficult.

In yeast, the Tor proteins are assembled into two complexes, known as TORC1 and TORC2. TORC1 consists of either kinase Tor1 or Tor2, Lst8, Kog1 and Tco89 (Hall, 1996). TORC1 controls proliferation by coupling nutrient sufficiency, including amino acid availability, with growth by activating anabolic processes such as protein synthesis while also repressing catabolic processes such as autophagy (Loewith and Hall, 2011). The TORC2 complex is comprised of Tor2 and Avo1, 2 and 3. TORC2 is insensitive to rapamycin and is responsible for cell integrity processes such as actin organization (Hall, 1996). In mammals these two complexes have similar roles as in yeast, however both complexes share the same TOR kinase (Loewith and Hall, 2011).
The regulatory pathway upstream of TORC1 is complex, and many aspects remain to be determined. The EGO complex has been identified as an important regulator of TORC1 (Binda 2009). In yeast the EGO complex is composed of four proteins: Ego1, Ego3, Gtr1, and Gtr2. Gtr1 and Gtr2 are Ras-family GTPases (Binda et al., 2009; Kim et al. 2008). Loss of Gtr1 results in reduced TORC1 activity along with slow growth and is suppressed by deletion of the TCO89 gene (Binda et al., 2009). This observation suggests that the EGO complex can both positively and negatively regulate TORC1 activity via Tco89 (Binda et al., 2009). The mechanism by which amino acids regulate TORC1 activity upstream remains mysterious but is thought to involve the EGO complex (Binda et al., 2009).

There is a network of relatively poorly characterized pathways downstream of TORC1, but to date one of the best-characterized targets downstream is the AGC kinase Sch9 (Powers, 2007). It has been shown that a total of six target sites in the C-terminus of Sch9 are phosphorylated by TORC1 (Powers, 2007). This phosphorylation only occurs in nutrient rich conditions (Powers, 2007), and consequently has made it a common readout of TORC1 activity. In addition to Sch9, TORC1 also regulates the PP2A phosphatase complex and the PP2A-like complex (Loewith and Hall, 2011; Piotr et al., 2002). The yeast PP2A catalytic subunit is encoded by PPH21 and PPH22 and forms a complex with the scaffolding subunit Tpd3 and a variable regulatory subunit of either Cdc55 or Rts1 (Sneddon et al., 1990; Ronne et al., 1991). The PP2A-like phosphatase is known as Sit4 (Di Como and Arndt, 1996; Piotr et al., 2002), which forms complexes with four Sit4-associated proteins (SAPs). These phosphatases were first identified as having a role downstream of TORC1 when a subpopulation of these enzymes were found to interact with the regulatory protein Tap42, in a TORC1-dependent manner (Di Como and Arndt 1996). Additionally, the PP2A complex was later found to exist as a heterodimer of
PP2A with Tap42 (Yu and Broach, 1999). In this association the target of TOR is thought to be Tap42, and it was found the TORC1 complex phosphorylates Tap42 under nutrient rich conditions (Yu and Broach, 1999). This phosphorylation is required for the interaction between Tap42 and the PP2A complex (Yu and Broach, 1999). This association is essentially autoregulated by the ability of the PP2A holoenzyme containing Tpd3 and Cdc55 to dephosphorylate Tap42 when necessary (Yu and Broach, 1999).

The nitrogen catabolite repression (NCR) sensitive GATA transcription activators Gln3 and Gat1 have become common reporters of Tor1 mediated gene regulation (Georis et al., 2008). Originally, the utility of GATA factor subcellular localization as a reporter of Tor signalling came from the observation that Gat1 and Gln3 respond similarly to rapamycin, which inhibits Tor, and to nitrogen starvation; they localize to the nucleus under these conditions and NCR-sensitive transcription increases (Loewith and Hall, 2011; Georis et al., 2008). It was thought that these transcription factors were regulated by similar mechanisms downstream of Tor signalling. However, recent studies revealed that Gat1 localization is independent of the Tor downstream phosphatase Sit4 and receptor protein Ure2, whereas Gln3 is heavily regulated by these proteins (Georis et al., 2008). The regulatory mechanism of Gat1 localization remains unclear, however, and consequently regulation of these two GATA factors seems to represent divergent downstream pathways of Tor, and more complicated than originally thought (Georis et al., 2008).

1.9 Hom3

*HOM3* encodes the aspartokinase in *S. cerevisiae* and is the first enzyme in the pathway that converts aspartate to methionine and threonine (Figure 2.) (Rafalksi and Falco, 1990). In bacteria and higher plants, the first step in the pathway that converts aspartate to lysine,
methionine and threonine is the activation of this substrate by phosphorylation, performed by an
aspartokinase (Rafalksi and Falco, 1990). In E. coli three aspartokinases exist whose activities
are inhibited by and have different sensitivities to the products lysine, threonine and methionine
(Rafalksi and Falco, 1990). However, in fungi, lysine is derived from a different pathway
involving α-ketoglutarate, thereby eliminating the need for multiple forms of aspartokinase
(Rafalksi and Falco, 1990). Yeast aspartokinase activity is inhibited by excess threonine, and
mutations in this enzyme cause threonine and methionine auxotrophy (Rafalksi and Falco, 1990).

It has been shown via a yeast-two hybrid assay that the Tor pathway member Fkbp12
(encoded by FPR1) is able to directly bind Hom3 in vivo and in vitro (Alarcon et al., 1997). As
mentioned above, this peptidyl-prolyl isomerase (Fkbp12) forms a complex with rapamycin
which binds to and inhibits TORC1 (Alarcon et al., 1997). It was shown that binding of
rapamycin to Fkbp12 abolished the interaction with Hom3 (Alarcon et al., 1997). Although hom3
mutants are auxotrophic for threonine and methionine, an fprl mutant lacking FKB12 showed
no sensitivity to threonine or methionine starvation (Alarcon et al., 1997). This provides
evidence that Fkbp12 is not needed for Hom3 stability or functionality (Alarcon et al., 1997).
Later, Hom3 having a role downstream of TORC1 was further supported by evidence that the
downstream TORC1 target Atg1 is likely the kinase that phosphorylates Hom3 (Oliviera et al.,
2015). These studies support that Hom3 may have a role in Tor signalling, however, the details
are not well understood (Alarcon et al., 1997; Oliviera et al., 2015).

1.10 Thesis Objectives

The overall aim of this project was to identify genes required for induction of the GAL
genes in a Cdk8-dependent manner, with the goal of finding upstream regulators of Cdk8, in
order to gain insight into this enzyme’s regulatory mechanisms. This was the objective of the
“gft” screen which produced a collection of mutants that are unable to induce long-term adaptation (LTA) of the GAL genes, which likely represent regulators of Cdk8 activity. My research project involved initial characterization of the gft mutants. I focused most attention on the gft1 mutation, which was determined to represent a recessive allele of hom3. Importantly, characterization of the hom3/gft1 allele revealed a previously unrecognized role of the TOR signalling pathway for GAL gene induction.
Figure 1. Model of GAL gene induction. A. In wildtype induction conditions Gal4 “4” is bound as a dimer to the UAS and inhibited by Gal80 “80”. The black bars within the boxes represent binding sites for Gal4 dimers. In the presence of galactose and Gal3 “3”, inhibition is relieved by binding of Gal3 to Gal80 and induction is further driven by phosphorylation of S699 on Gal4 by Cdk8. B. Induction under gal3 or long-term adaptation conditions requires phosphorylation of S699 by Cdk8 and is thought to then be sustained by production of Gal1 “1” which mimics Gal3.
Figure 2. Metabolic pathway converting Aspartate into Threonine and Methionine in yeast. A diagram of the synthesis of threonine and methionine is shown. The genes, enzymes, and metabolic intermediates relevant to this study are shown.
Chapter 2: MATERIALS AND METHODS

2.1 Yeast strains and plasmids

Strains used for these experiments are listed in Table 1, and all gft mutant strains are described in Table 2. All gene disruptions and integrations were made by homologous recombination at each selected chromosomal loci using standard PCR-based methods and confirmed by PCR, all primers can be found in Table 3 (Longtine et al., 1998). Standard methods and media were used for yeast growth and transformation. Two percent galactose in the media was used to induce the expression of genes controlled under the GAL1 promoter. Synthetic minimal media with appropriate amino acid supplements was used for cells containing plasmids. All plasmids used are listed in Table 4. The Hom3 kinase inactive expression plasmid was generated by site-directed mutagenesis using the Q5 Site-Directed Mutagenesis kit (NEB) following manufacturer’s protocols and verified by sequencing.

2.2 Fluorescent Microscopy Sample Preparation

Strains W303-1A, ISY128 and 1471 were transformed with plasmid pRS416GAT (0.5 μg) which expresses GAT1-GFP protein. In addition, W303-1A was transformed with the plasmid pJP015 (0.7 μg) which expresses CDK8-GFP for use as a nuclear localization control; both plasmids have a URA3 marker and ARS-CEN replicon. Cells were grown SD lacking uracil overnight at 30°C with shaking, and diluted to 0.1 OD A600, and incubated for a further 4 hours at 30°C until log phase was reach with an OD A600 of approximately 0.8. 5 μL of Hoeschst-3342 working solution (10 μL stock:990 μL dH2O) was then added to 1 mL of each culture. Samples were incubated for 30 minutes at 25°C in a roller-drum, protected from the light. The cells were the spun at 2000 rpm for 4 minutes at room temperature, and most of the supernatant was
removed. Immediately prior to imaging, the cells were resuspended and 2 μL was placed on a
slide and topped with a cover slip, prior to examining by microscopy.

2.3 Transformation (PCR fragments)

The PCR fragments for gene disruption generated using standard protocols were
transformed using a high efficiency transformation protocol. 5 mL of YPD (1% Bacto yeast
extract, 2% Bacto peptone, 2% Dextrose) was inoculated with the W303-1A-derived strain and
incubated overnight at 30°C with shaking. A 5-fold dilution was made into fresh YPD and grown
at 30°C for 5 hours. Cells were pelleted, washed in 10 mL TL (10 mM Tris-HCl pH 7.5, 1 mM
EDTA, 100 mM LiOAc), and resuspend in 2 mL TL. Tubes were placed in a tightly packed
bucket full of ice, and left overnight; the next morning tubes were gently inverted several times
to resuspend cells and placed into fresh ice. For each transformation 100 μL of competent cells
were aliquoted into sterile tubes and 50 μg of sheared salmon sperm carrier DNA was added.
Approximately 1 μg of each PCR fragment was added and mixed gently by pipetting and
incubated at 30°C for 30 minutes. Then 400 μL of TLP (10 mM Tris-HCl pH 7.5, 1 mM EDTA,
100 mM LiOAc, 44% PEG 4000) was added to each transformation and incubated at 30°C for
another 60 minutes. Cells were then heat shocked for 5 minutes at 42°C, placed on ice for 2
minutes, and spread on YPD plates and incubated at 30°C for 24 hours. Transformants were then
replica plated onto YPD+G418 selection plates (200 μg/mL G418 Life Technologies).
Approximately 8 single colonies per transformation were then selected and re-streaked onto
YPD+G418 plates via a wooden toothpick.
2.4 Transformation (Plasmids)

The transformation protocol for plasmids consisted of growing a 5 mL overnight culture at 30°C of the specific yeast strain in the applicable synthetic selection media. For each transformation 2 mL of the desired culture was harvested by centrifugation. The cells were then resuspended in 360 μL Transformation Mix which consisted of 33% PEG 3500, 0.1 M LiOAc, 270 ng sheared salmon sperm carrier DNA (heated and vortexed), and approximately 1 μg plasmid DNA and dH₂O to final volume. The transformations were incubated at 42°C for 90 minutes, microcentrifuged at 13,000 rpm for 30 seconds; the supernatant was removed and the cells resuspended in 1 mL dH₂O. Approximately 400 μL was spread on the appropriate dropout selection plates, and incubated at 30°C.

2.5 Genomic DNA Preparation

Yeast were grown overnight in YPD and 1.5 mL were pelleted at 4000 rpm for 3 mins. The cells were resuspended in 200 μL of breaking buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl [pH 8.0], 1 mM EDTA). Next 200 μL of glass beads, and 200 μL of phenol/chloroform/isoamyl alcohol was added and the samples were vortexed for 3 mins. Then, 200 μL of TE was added and the samples were vortexed briefly before centrifugation at 13000 rpm for 5 mins. The aqueous layer was transferred to a new tube, 1 mL of 95% ethanol was added, and the tubes inverted several times to mix. Samples were spun at 13000 rpm for 5 mins, and the supernatant removed, the tubes spun again for a 10 secs and residual ethanol was removed. The tubes were left to stand at room temp for 15 mins, or the tubes were placed in a Speedvac for several minutes to dry the DNA pellets. The DNA was resuspended in 300 μL TE, and then used for PCR reactions to screen for insertions or disruptions.
2.6 *In vitro* TORC1 Kinase Assay

Samples for the TORC1 kinase assay were prepared as previously described in (Tanigawa 2017) with some minor adjustments. Spheroplasts were prepared from logarithmically growing cells growing in 50 mL of YPD and harvested by centrifugation (1500xg, 5min) and washed once with ice cold milliQ dH$_2$O containing 2 mM PMSF. The pellets were resuspended in 1 mL 0.1 M Tris-HCL (pH 9.4), 10 mM DTT, then incubated for 10 minutes. The cells were spun down (1500xg, 2 min) and then resuspended in 1 mL of spheroplasting buffer (0.7M Sorbitol, 10mM Tris-HCl {pH 7.5}, 1mM DTT, 20mM NaN$_3$, and 0.1 mg/mL Zymolase). The mixtures were incubated for 20-30 minutes at room temperature before centrifuging at 1000xg for 2 min at 4°C. The pellets were washed once with 1 mL cold sorbitol buffer (1M sorbitol, 150 mM K acetate, 5 mM Mg acetate, 20 mM HEPES-KOH [pH 6.6]), and resuspended in sorbitol buffer to an OD A$_{600nm}$ of 100 per mL. The spheroplasts were stored at -80°C in 20 μL aliquots until use. To prepare semi-intact cells, spheroplast aliquots were thawed for 1 min at 30°C 1 mL of buffer A was added (0.25M sorbitol, 150 mM K acetate, 5 mM Mg acetate, 20 mM HEPES-KOH [pH 6.6] and 200 ug/mL PMSF), and the samples incubated on ice for 5 min, prior to spinning at 13,000xg for 1 min. The pellets were resuspended in 1 mL buffer A, incubated for 5 mins on ice and spun again. The pellets were washed a further time with buffer A, and then once with buffer B (0.05 M sorbitol, 150 mM k acetate, 5 mM Mg acetate, 20 mM HEPES-KOH [pH6.6], and 200 ug/mL PMSF), and then a final wash with buffer A before the semi-intact cells were resuspended in import buffer (0.4M sorbitol, 150 mM K acetate, 5mM Mg acetate, 20 mM HEPES-KOH [pH6.6]) at 0.7 OD/mL. For kinase assays, the semi-intact cells were resuspended in 18 μL reaction buffer (0.4M sorbitol, 150 mM k acetate, 5mM Mg acetate, 20 mM HEPES-KOH [pH 6.6], 40 mM creatine phosphate, 200 ng/ul creatine
kinase, 1 mM Pefabloc SC, 4 ug/mL aprotinin, 1 ug/mL pepstatinA, 2 ug/mL leupeptin, 0.3 ug/mL 4EBP1). The reactions were started by adding 2 μL of ATP-amino acids (5 mM ATP with 2 % amino acid mix) and incubated for 10 minutes at 30°C. Reactions were stopped by adding 20 μL of 2X SDS buffer and boiled for 4 minutes. Samples were centrifuged at 9,000 rpm for 2 min and the supernatant was analysed by SDS-PAGE and immunoblotting with anti-4EBP1 antibodies (Cell Signalling Lot 10) and anti-Phospho-4EBP1 (Cell signalling Lot 10).

2.7 Western Blot Analysis

Protein samples were heated at 95°C for 3 minutes in 1X Laemmli buffer and separated via SDS polyacrylamide gel electrophoresis (SDS-PAGE) using 10% polyacrylamide (Resolving gel: 10% Acrylamide/Bis 29:1, 0.1% SDS, 0.4 M Tris pH 8.8, 0.1% Ammonium Persulphate, 0.06% TEMED; Stacking gel: 5% Acrylamide/Bis 29:1, 125 mM Tris pH6.8, 0.1% SDS, 0.1% APS, 0.02% TEMED). The polyacrylamide gels were cast in 1.0 mm thick glass plates with 15-well combs. For each lane 10 μL of sample was added and the gel was run at 115V for 15 minutes, and then 185V for 60 minutes. Nitrocellulose membrane and filter paper were soaked in transfer buffer (118 mM Tris, 191 mM Glycine, 20% methanol) for 10 minutes prior to assembling in the transfer apparatus. Following SDS-PAGE, the polyacrylamide gel was soaked in transfer buffer for 10 minutes. The proteins were then transferred to the nitrocellulose membrane by wet electrophoretic transfer at 100V for 120 minutes. The membrane was blocked with 2% milk for 60 minutes, washed 4x5 minutes with PBS-T (PBS + 0.05% Tween-20), then incubated at 4°C overnight with the primary antibody in 2% skim milk PBS+T. The membrane was then washed 4x5 minutes with PBS-T, and then incubated with a secondary HRP conjugated antibody at room temperature for 60 minutes. Following incubation, the membrane was washed
4x5 minutes with PBS-T and the signal was developed with the SuperSignal West Femto chemiluminescent substrate (Thermo Fisher).

2.8 In vitro Kinase Assays

Yeast expressing 3x-FLAG-Srb10 were grown in SD-Ura containing 2% glucose were grown overnight at 30°C. The cells were diluted into 200 mL SD-Ura to an OD A600nm = 0.1 and allowed to grow for 4 hours until and OD A600nm = 0.6 – 0.8 was reached. Next, 50 mL of culture was pelleted and the cells were lysed in kinase lysis buffer (KLB) (50 mM Tris [pH7.5], 5mM ETDA, 200mM NaCl, 0.1% NP-40, and protease inhibitors) by vortexing with glass beads. FLAG-tagged Srb10 was recovered by immunoprecipitation with anti-FLAG-conjugated M2 agarose beads (Sigma, Lot 7467VC). Samples were washed two times in KLB followed by two times in kinase buffer (KB) (10 mM MgCl2, 50 mM Tris [pH 7.5], 1 mM DTT and protease inhibitors). Kinase reactions were performed in 10 μL KB with 1 μg substrate, diluted in KB to a final volume of 10μL. Reactions were started by adding 2 pmol [γ-32P]ATP to the reactions and incubating at 30°c for 20 minutes. Reactions were then resolved on 10% SDS PAGE and visualized by exposure to Kodak Biomax film.

2.9 Dilution Plate Growth Assays

Yeast strains were first grown overnight at 30°C in appropriate media. Cultures were normalized to an OD600 of 1 and spotted in 10-fold serial dilutions onto plates supplemented with either 2% glucose and rapamycin at 5 ng/mL, 5% formamide, .5% MMS, 60 ug/mL hygromycin B, or 2% galactose and containing ethidium bromide at 20 mg/L, as indicated.

2.10 Flow Cytometry

Yeast strains were grown overnight at 30°C in synthetic complete media supplemented with glycerol, lactic acid and ethanol. The cultures were induced with 2% galactose, and at the
indicated time points samples of 400,000 cells were diluted in PBS to a final volume of 1 mL. Samples were then analyzed using the Guava Easycyte Flow Cytometer (MilliporeSigma) to determine mean fluorescence intensity (MFI), using FlowJo software.

2.11 Mating, Sporulation and Tetrads Analysis

Yeast strains for mating were grown overnight in patches on YPD at 30°C. A loopful of the complementary strain was suspended in 1 mL YPD, from an overnight culture, and 250 μL was spread onto YPD and allowed to dry. Plates with patched strains were replica plated onto a lawn of cells of the complementary mating type and the plates were incubated at 30°C for 4 hours. The plates were then replica plated onto media selective for diploids and incubated at 30°C.

Sporulation was performed by first culturing diploids for 2 days on BMG media (2% peptone, 1% yeast extract, 4x10^{-5} % biotin, 1.34% yeast nitrogen base, 0.1 M potassium phosphate buffer [pH 6.0], and 1% glycerol). A loopful of the cells was then inoculated into 5 mL 1% potassium acetate and incubated with gentle shaking for 2 days. When asci developed, 1 mL of the culture was pelleted by centrifugation for 30 seconds and resuspended in 500 μL TE sorbitol. 5μL of β-glucuronidase (Sigma) and 8 μL BME was added and allowed to digest at 30°C for 15 minutes. Digested asci were streaked onto a level YPD plate and dissected using a Zeiss microscope with Singer micromanipulator. Spores were germinated on YPD and grown for 2 days before being replica plated onto EB-Gal plates and grown for a further 5 days.
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</tr>
<tr>
<td></td>
<td>gal3::LEU2, hom6::kanMX6</td>
<td></td>
</tr>
<tr>
<td>yNH016</td>
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<td>This Study</td>
</tr>
<tr>
<td></td>
<td>tco89::kanMX6</td>
<td></td>
</tr>
<tr>
<td>STRAIN</td>
<td>GENOTYPE</td>
<td>SOURCE</td>
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<tr>
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<tr>
<td>yNH019</td>
<td>MATa, ade2, can1, his3, leu2, trp1, ura3, gal3::LEU2, tor1::kanMX6, cdc55::His3MX6</td>
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<tr>
<td>yNH024</td>
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<tr>
<td>yNH025</td>
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<td>This Study</td>
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<tr>
<td>yNH026</td>
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<tr>
<td>yNH028</td>
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<td>This Study</td>
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<td>yNH029</td>
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<td>This Study</td>
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<td>This Study</td>
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<tr>
<td>yNH031</td>
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<td>This Study</td>
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<tr>
<td>yNH032</td>
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<td>This Study</td>
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<tr>
<td>yNH033</td>
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<td>This Study</td>
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<td>yNH034</td>
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<td>This Study</td>
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<tr>
<td>yNH035</td>
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<tr>
<td>yNH036</td>
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<td>This Study</td>
</tr>
<tr>
<td>yNH037</td>
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<td>This Study</td>
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<tr>
<td>YJR40</td>
<td>MATa, trp, ura, leu, his, gal4 del, gal3::LEU2, GAL1-lacZ::URA3</td>
<td>J. Rohde</td>
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Table 2: List of *gft* mutant strains isolated from screen

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>GENOTYPE</th>
<th>SOURCE</th>
</tr>
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<tr>
<td>ISY135</td>
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<td>I. Sadowski</td>
</tr>
<tr>
<td>ISY158</td>
<td>MATa, HIS3, <em>can1</em>, <em>ade2</em>, <em>leu2</em>, <em>trp1</em>, <em>ura3</em>, <em>gal3::LEU2</em>, <em>gft2-1</em></td>
<td>I. Sadowski</td>
</tr>
<tr>
<td>ISY159</td>
<td>MATa, HIS3, <em>can1</em>, <em>ade2</em>, <em>leu2</em>, <em>trp1</em>, <em>ura3</em>, <em>gal3::LEU2</em>, <em>gft3-1</em></td>
<td>I. Sadowski</td>
</tr>
<tr>
<td>ISY160</td>
<td>MATa, HIS3, <em>can1</em>, <em>ade2</em>, <em>leu2</em>, <em>trp1</em>, <em>ura3</em>, <em>gal3::LEU2</em>, <em>gft4-1</em></td>
<td>I. Sadowski</td>
</tr>
<tr>
<td>ISY146</td>
<td>MATa, HIS3, <em>can1</em>, <em>ade2</em>, <em>leu2</em>, <em>trp1</em>, <em>ura3</em>, <em>gal3::LEU2</em>, <em>gft5-1</em></td>
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</tr>
<tr>
<td>ISY161</td>
<td>MATa, HIS3, <em>can1</em>, <em>ade2</em>, <em>leu2</em>, <em>trp1</em>, <em>ura3</em>, <em>gal3::LEU2</em>, <em>gft6-1</em></td>
<td>I. Sadowski</td>
</tr>
<tr>
<td>ISY162</td>
<td>MATa, HIS3, <em>can1</em>, <em>ade2</em>, <em>leu2</em>, <em>trp1</em>, <em>ura3</em>, <em>gal3::LEU2</em>, <em>gft7-1</em></td>
<td>I. Sadowski</td>
</tr>
<tr>
<td>ISY163</td>
<td>MATa, HIS3, <em>can1</em>, <em>ade2</em>, <em>leu2</em>, <em>trp1</em>, <em>ura3</em>, <em>gal3::LEU2</em>, <em>gft8-1</em></td>
<td>I. Sadowski</td>
</tr>
<tr>
<td>ISY164</td>
<td>MATa, HIS3, <em>can1</em>, <em>ade2</em>, <em>leu2</em>, <em>trp1</em>, <em>ura3</em>, <em>gal3::LEU2</em>, <em>gft9-1</em></td>
<td>I. Sadowski</td>
</tr>
<tr>
<td>ISY165</td>
<td>MATa, HIS3, <em>can1</em>, <em>ade2</em>, <em>leu2</em>, <em>trp1</em>, <em>ura3</em>, <em>gal3::LEU2</em>, <em>gft10-1</em></td>
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</tr>
<tr>
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<td>MATa, HIS3, <em>can1</em>, <em>ade2</em>, <em>leu2</em>, <em>trp1</em>, <em>ura3</em>, <em>gal3::LEU2</em>, <em>gft11-1</em></td>
<td>I. Sadowski</td>
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<tr>
<td>ISY167</td>
<td>MATa, HIS3, <em>can1</em>, <em>ade2</em>, <em>leu2</em>, <em>trp1</em>, <em>ura3</em>, <em>gal3::LEU2</em>, <em>gft12-1</em></td>
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<tr>
<td>ISY168</td>
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<td>ISY169</td>
<td>MATa, HIS3, <em>can1</em>, <em>ade2</em>, <em>leu2</em>, <em>trp1</em>, <em>ura3</em>, <em>gal3::LEU2</em>, <em>gft13-2</em></td>
<td>I. Sadowski</td>
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<tr>
<td>ISY170</td>
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<td>I. Sadowski</td>
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<tr>
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</tr>
<tr>
<td>ISY187</td>
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<td>I. Sadowski</td>
</tr>
<tr>
<td>ISY188</td>
<td>MATa, HIS3, <em>can1</em>, <em>ade2</em>, <em>leu2</em>, <em>trp1</em>, <em>ura3</em>, <em>gal3::LEU2</em>, <em>gft3-1</em></td>
<td>I. Sadowski</td>
</tr>
<tr>
<td>ISY189</td>
<td>MATa, HIS3, <em>can1</em>, <em>ade2</em>, <em>leu2</em>, <em>trp1</em>, <em>ura3</em>, <em>gal3::LEU2</em>, <em>gft4-1</em></td>
<td>I. Sadowski</td>
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<tr>
<td>ISY190</td>
<td>MATa, HIS3, <em>can1</em>, <em>ade2</em>, <em>leu2</em>, <em>trp1</em>, <em>ura3</em>, <em>gal3::LEU2</em>, <em>gft5-1</em></td>
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<tr>
<td>ISY191</td>
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<td>I. Sadowski</td>
</tr>
<tr>
<td>ISY192</td>
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<td>I. Sadowski</td>
</tr>
<tr>
<td>ISY193</td>
<td>MATa, HIS3, <em>can1</em>, <em>ade2</em>, <em>leu2</em>, <em>trp1</em>, <em>ura3</em>, <em>gal3::LEU2</em>, <em>gft8-1</em></td>
<td>I. Sadowski</td>
</tr>
<tr>
<td>ISY194</td>
<td>MATa, HIS3, <em>can1</em>, <em>ade2</em>, <em>leu2</em>, <em>trp1</em>, <em>ura3</em>, <em>gal3::LEU2</em>, <em>gft9-1</em></td>
<td>I. Sadowski</td>
</tr>
<tr>
<td>ISY195</td>
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<td>I. Sadowski</td>
</tr>
<tr>
<td>ISY196</td>
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<td>I. Sadowski</td>
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<tr>
<td>ISY197</td>
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<tr>
<td>ISY198</td>
<td>MATa, HIS3, <em>can1</em>, <em>ade2</em>, <em>leu2</em>, <em>trp1</em>, <em>ura3</em>, <em>gal3::LEU2</em>, <em>gft13-1</em></td>
<td>I. Sadowski</td>
</tr>
<tr>
<td>ISY199</td>
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<td>I. Sadowski</td>
</tr>
<tr>
<td>ISY200</td>
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</tr>
<tr>
<td>ISY201</td>
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<td>I. Sadowski</td>
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Table 3: List of plasmids used in this study

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<thead>
<tr>
<th>PLASMID</th>
<th>USE</th>
<th>SOURCE</th>
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<tr>
<td>pFA6a-KanMX6</td>
<td>Create disruptions with PCR products using kanMX6 cassette</td>
<td>M. Longtine</td>
</tr>
<tr>
<td>pFA6a-HIS3MX6</td>
<td>Create disruptions with PCR products using His3MX6 cassette</td>
<td>M. Longtine</td>
</tr>
<tr>
<td>pFA6a-TRP1-pGAL1-GFP</td>
<td>Construction of GAL-inducible GFP-tagged integrated fusions</td>
<td>M. Longtine</td>
</tr>
<tr>
<td>pRS314</td>
<td><em>TRP1</em> marker; single copy yeast plasmid</td>
<td>P. Hieter</td>
</tr>
<tr>
<td>pIS556</td>
<td><em>TRP1</em> marker; Expressing HOM3-3X FLAG-6His</td>
<td>I. Sadowski</td>
</tr>
<tr>
<td>pIS601</td>
<td><em>TRP1</em> marker; ADH1 promoter expressing N-term 3X-FLAG fusion with HOM3.</td>
<td>I. Sadowski</td>
</tr>
<tr>
<td>pIS574</td>
<td><em>TRP1</em> marker; TEF1 promoter expressing HOM3 ORF not fused to an epitope tag.</td>
<td>I. Sadowski</td>
</tr>
<tr>
<td>pIS297</td>
<td><em>TRP1</em> marker; Clone 1 from complementation of swg1/gft1 mutation.</td>
<td>I. Sadowski</td>
</tr>
<tr>
<td>pIS484</td>
<td><em>URA3</em> marker; TEF1 promoter expressing Srb10-3X-FLAG-6his C-terminal tagged including 14 N-terminal residues upstream of the in frame Met translation start</td>
<td>I. Sadowski</td>
</tr>
<tr>
<td>pRD038</td>
<td>YCpG4Trp A699 GAL4</td>
<td>R. Dhanawansa</td>
</tr>
<tr>
<td>YCpG4trp</td>
<td>GAL4 expressed off of its own promoter in an ARS-CEN TRP1 vector.</td>
<td>I. Sadowski</td>
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Table 4: List of primers used in this study

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<th>PRIMER</th>
<th>SEQUENCE (5’-3’)</th>
<th>DETAILS</th>
</tr>
</thead>
<tbody>
<tr>
<td>IS2399</td>
<td>AGACTGTCAAGGAGGGTTATTCT</td>
<td>Reverse primer in the TEF1 promoter to check disruptions</td>
</tr>
<tr>
<td>IS1709a</td>
<td>ATAGTAGCTTCACGAGAGAGTCATTTGGTAA GCAAACCATACATCAACCCGGCTAGCAGGTTT GCATGATCGGATCCCCGGAATTAA</td>
<td>Forward primer to amplify marker for disruption of tor1</td>
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<tr>
<td>IS1710a</td>
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<td>Reverse primer to amplify marker for disruption of tor1</td>
</tr>
<tr>
<td>IS2465</td>
<td>GAGGACTTCTTTTGTATTGGAAGGTGC</td>
<td>Forward primer to check tor1 disruption</td>
</tr>
<tr>
<td>IS2475</td>
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</tr>
<tr>
<td>IS2476</td>
<td>ATCTATTTATATATATATAATACCTATGTAAAA ATATGTCTGTTTTACTGATGAATTCGAGCTCGTTAAAC</td>
<td>Reverse primer to amplify marker for disruption of hom6</td>
</tr>
<tr>
<td>IS2477</td>
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</tr>
<tr>
<td>IS2497</td>
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</tr>
<tr>
<td>IS2498</td>
<td>TTTACCTCGAAAGCATATAGCTACTCTTTTAA CTGTGTGCTCGGTGTTTGTTGTTGAATTCAA CTCGTTAAAC</td>
<td>Reverse primer to amplify marker for disruption of tco89</td>
</tr>
<tr>
<td>IS2499</td>
<td>AACGACTAATTCACACGTTTGTTTATTCAA</td>
<td>Forward primer to check tco89 disruption</td>
</tr>
<tr>
<td>IS2325</td>
<td>AGGTCAAACCTGGAGAGATCTACGCATAAAAG AAATATAATATAGCGACACGGATCCCCCGGT TAAATTAA</td>
<td>Forward primer to amplify marker for disruption of cdc55</td>
</tr>
<tr>
<td>PRIMER</td>
<td>SEQUENCE (5’-3’)</td>
<td>DETAILS</td>
</tr>
<tr>
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<td>-------------------------------------------------------------------------</td>
</tr>
<tr>
<td>IS2326</td>
<td>GAATTCAAGTTCAATTATAATTCAATTTAAAACAGTAGTGATATGTTGGGAATTCCAGCTCGT TTAAAC</td>
<td>Reverse primer to amplify marker for disruption of <em>cdc55</em></td>
</tr>
<tr>
<td>IS2022</td>
<td>TTCTAGAAGAATTCCGATTACGTTAAC                                                                -----------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>Forward primer to check <em>cdc55</em> disruption</td>
</tr>
<tr>
<td>IS2554</td>
<td>TCGAATGAGTGACTCGTTTGCTAGTGACTCTG ACCTGTTTTTATTCCAGCAAGAGGAATTCCAGGCTCGTTAAAC</td>
<td>Forward primer to amplify GAL-GFP for disruption of <em>ade8</em></td>
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<tr>
<td>IS2555</td>
<td>GATGTTTCGCAGCTCTCACTTTGAAGAATGCCA AATATAAAAGTATAAAATATGGGAACTCCCTAGCGGATCTGC</td>
<td>Reverse primer to amplify GAL-GFP for disruption of <em>ade8</em></td>
</tr>
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<td>IS2569</td>
<td>CTTCTAAAGTTGAATAACGCTCCTAG</td>
<td>Forward primer to check disruption at <em>ade8</em></td>
</tr>
<tr>
<td>IS2328</td>
<td>GGCTATACCGcaTTATGTGCGG</td>
<td>Forward primer to create <em>HOM3</em> D237A KD mutant</td>
</tr>
<tr>
<td>IS2329</td>
<td>ACGACCAACACCATTCAG</td>
<td>Reverse primer to create <em>HOM3</em> D237A KD mutant</td>
</tr>
<tr>
<td>IS2634</td>
<td>TCCCTCTGATTCAGTGCC</td>
<td>Forward primer to sequence <em>HOM3</em> D237A mutants</td>
</tr>
<tr>
<td>IS2635</td>
<td>ATGTATAACTTCGGAACCATAATATGTTAATTTC</td>
<td>Reverse primer to sequence <em>HOM3</em> D237A mutants</td>
</tr>
</tbody>
</table>
Chapter 3: RESULTS

3.1 Characterization of the gft Mutant Phenotypes

3.1.1 The gft Mutant Screen

This screen was conceptualized after the discovery that Cdk8 acts similar to a throttle for the GAL genes and was shown to be required for full induction in yeast defective for gal3 (Rohde et al., 2000). This led to the model that Cdk8 modulated GAL induction in response to environmental conditions. The objective of the screen was to identify components involved in the Cdk8-dependent activation of Gal4, with the goal of determining the signalling pathway(s) that control Cdk8 kinase activity. The screen was similar to a synthetic lethal screen, however a standard synthetic genetic assay (SGA) could not be used due to there being an ill-defined difference in the GAL regulon in the deletion collection BY4741 background strain which inhibits LTA (Mortimer and Johnston, 1986). The screen was based on the observation that gal3 srb10 yeast are completely incapable of inducing GAL gene expression, even by the previously described LTA pathway, and are unable to utilize galactose as the sole source of carbon, which is most stringently and simply assayed by growth on plates containing ethidium bromide, which inhibits mitochondrial function and the ability to utilize any molecule other than six-carbon sugars as a source of carbon. With this rationale, mutations affecting upstream regulatory components of Cdk8 should cause a defective growth phenotype on EB-Gal in combination with a gal3 null mutation.

The screen work flow is outlined in Figure 3 and was carried out briefly as follows. A W303-derived MATa gal3 strain was mutagenized by UV irradiation, with a survival rate of ~50%, and allowed to form colonies on minimal media containing glycerol as the carbon source (Fred, 2008). Once colonies had formed they were replica-plated onto media containing
galactose as the sole carbon source (EB-galactose). Forcing the mutagenized yeast to form colonies on glycerol media before replica plating to EB-galactose was performed to eliminate mutations affecting respiration which would result in a Gal- phenotype, as discussed above, and to ensure a sufficient number of cells would be transferred because gal3 yeast grow poorly on EB-galactose (Rotman et al., 1953). After 5 days of growth the EB-galactose plates were compared to the initial glycerol plates, and isolates that could not form colonies on EB-gal were selected. The initial isolates were expanded and transformed with a plasmid bearing wild type GAL3, and the transformants plated onto EB-galactose plates; those which did not grow on EB-gal here, representing ~50% of the initial isolates, were discarded, as these must represent mutations in one of the known GAL regulon genes, as described above. Isolates which only produced a gal phenotype on EB-gal plates when also defective for gal3 were mated to a MATα gal3 strain to produce diploids. The diploids were sporulated and subjected to tetrad analysis; those where the growth defect on EB-galactose segregated 2:2 were analysed further. MATα derivatives from the initial cross were also used in mating reactions with the full set of other mutants for complementation analysis. This effort resulted in 16 complementation groups, which were designated the gal four throttle (gft) mutants.

3.1.2 Characterization of the gft Mutant Phenotypes

The gft mutants isolated from the genetic screen described above were examined for growth under various conditions. Because the identity of most gft mutants were unknown when I started my research, and whole genome re-sequencing revealed no clear answers, we decided to screen the mutant collection under a variety of conditions to identify additional phenotypes that might provide insight towards identity of the mutants (Table 5). The hope was to group mutants with similar phenotypes with the expectation that these may represent genes in common
pathways upstream of Cdk8 activity. The phenotypes were qualitatively assessed with a range from WT GFT (gal3) growth or activity designated “++++” and no growth being “-”. Since the original screen utilized ethidium bromide added to galactose plates, this condition was assessed again individually for each of gft mutants. Ethidium bromide inhibits the mitochondrial function, forcing them to use the fermentable carbon source galactose, limiting survival to those than can properly induce their GAL genes. Unfortunately, because the starting background/wild type gal3 strain used for the original screen already grow quiet slowly and sparsely on EB-Gal, the gft phenotype for some of the mutants could be described as subtle. Consequently, we were interested in additional conditions that might produce stronger phenotypes for determining the identity of the gft mutants.

Since it has been established that Gal4 activity and induction of the GAL genes can occur by a Gal3-galactose mechanism, that is modified by a Cdk8-dependent pathway, the goal of this screen was to utilize the Cdk8-dependent pathway to uncover regulators of Cdk8 (Rohde et al., 2000). Obviously, it was important to determine whether Cdk8 activity was altered in the gft mutants. To determine this, an in vitro kinase assay was performed with each gft mutant strain, where FLAG-tagged Cdk8 was expressed, recovered by immunoprecipitation, and used in in vitro reactions with purified Gal4 protein as substrate. This analysis revealed that 6 of the 16 gft mutants showed altered Cdk8 activity as determined by weak phosphorylation of the Gal4 substrate. The gft mutants with altered Cdk8 activity were numbers 6, 9, 11, 13-2, 14 and 15; however, the identity of these mutants remains unknown.

The mutant collection was also screened for growth on sub-lethal concentrations of rapamycin which would indicate defects in Tor pathway function. This analysis revealed that gft 1, 5, 6, 7, 13-2, and 14 were highly sensitive to sub-lethal concentrations of rapamycin, and that
gft 11 and 13-1 were somewhat less sensitive. I discuss the significance of these observations below. We also examined effects of the gft mutants on growth in the presence of DNA damaging agents (DDAs), which can also indicate defects in transcriptional regulation; the DDAs chosen for this analysis were methyl methanesulfonate (MMS) and hydroxyurea (HU). Lastly, it was previously shown that some mutations in genes encoding components of Mediator, the Med proteins, are sensitive to sub-lethal concentrations of Hygromycin B (Barreto et al., 2011). As mentioned above, Mediator is a key component of transcription regulation, and consequently the gft mutants were screened for Hygromycin B sensitivity with the expectation that some would produce phenotypes similar to Med proteins (Buratowski 1999). This analysis revealed that the gft3, 5, 9 and 13-2 mutants were sensitive to sublethal concentrations of Hygromycin B.

3.2 A GAL-GFP reporter reveals the long-term adaptation phenotype (LTA) of gal3 yeast strains.

Yeast lacking gal3 are able to induce their GAL genes in a Cdk8-dependent manner, but at a much slower rate than wild type yeast, in a phenotype known as LTA. As previously mentioned above the phenotype of gal3 yeast on EB-Gal plates can be quite subtle. Consequently, I created a more quantitative GAL inducible GFP reporter system to measure LTA. With this reporter, I found that wild type yeast induce GFP expression within 1 hour post induction with 2% galactose, and after 4 hours 96% of cells had induced GAL expression, as shown by the increase in GFP expression (Figure 4). In contrast, and consistent with previous observations using LacZ reporter gene assays, only 24 hours post induction did the gal3 yeast begin to induce GAL expression. Furthermore, after 96 hours post induction only 11% of gal3 yeast expressed GFP, demonstrating a phenotype similar to that previously described as LTA for the GAL genes (Figure 5). Important to note, although induction by this Cdk8-dependent
pathway takes much longer than wildtype cells, the *gal3* yeast that do induce expression do so to the same extent as wildtype cells as seen by the GFP mean fluorescent intensity (MFI) of around $10^4$. This result aligns with what is seen when comparing growth of wild type cells versus *gal3* yeast when plated on EB-galactose (Figure 6). The *gal3* yeast are able to form far fewer colonies, yeast however the colonies that are formed, grow to a size similar to that of the wild type yeast.

### 3.3 A med2 disruption produces the gft phenotype; gft13-2 is allelic to med2.

Most of the *gft* mutants identified in the original screen remain unidentified, and consequently, because several of the mutants showed defects in growth on hygromycin B, a phenotype previously associated with defects in the mediator, I examined whether any of the *gft* mutants might represent genes encoding components of the mediator complex. As mentioned above, the mediator is characterised for its involvement in regulating transcription but also because it is known to interact directly with the kinase of interest Cdk8 (Buratowski, 1989; Hirst, 1999). For this purpose, the components of the middle module of mediator, including Med1 and Med9 were assessed along with the tail module component Med2. The Cdk8-module of Mediator is known to bind Mediator at the middle module (Tsai 2014). To examine their relationship to this phenotype, disruptions of either *med1*, *med2* or *med9* were made in W303 strains lacking *gal3*. These strains were screened for their ability to induce their *GAL* genes by growth on EB-Gal plates (Figure 7A). The *med1* and *med9* mutants were found to grow much like the wild type *gal3* strain and show the typical spotty LTA phenotype. However, the *med2 gal3* double mutant was unable to grow on EB-galactose, suggesting it is unable to induce the *GAL* genes which indicates a *gft*-like phenotype. The *med2* mutant was also found to be sensitive to sublethal concentration of hygromycin B, similar to several strains of the *gft* mutant collection, therefore I examined whether this gene may be allelic with any of the *gft* mutants (Barreto et al., 2011).
Because the \textit{gal3 \textit{gft3} and 13-2} mutants showed the strongest sensitivity to sublethal concentrations of hygromycin B, I mated these \textit{gft gal3} mutants to my \textit{gal3 med2} strain to produce diploids, which were then sporulated, and individual spores isolated from tetrads and assessed for growth on EB-Gal. It would be expected that if the \textit{gft} mutant was allelic to the \textit{med2} disruption, all 4 spores from each tetrad would be incapable of inducing their \textit{GAL} genes and be incapable of growth on EB-Gal (Figure 7B). The \textit{gal3 med2} to \textit{gal3 gft3} mating produced a 1:3 \textit{gft} phenotype (Figure 7B). In contrast, tetrads produced by sporulating a diploid produced by mating an otherwise wild type \textit{gal3} strain with the \textit{gal3 med2} deletion strain produced a 2:4 \textit{gft} phenotype, as expected. This analysis suggests that the \textit{med2} disruption is likely allelic with \textit{gft13-2}, as I observed a 4:0 \textit{gft} phenotype on EB-Gal from this mating.

\textbf{3.4 Characterization of the \textit{gft1} Mutant; a role for Tor signalling in regulation of \textit{GAL} gene induction.}

Several previous observations from our laboratory had indicated that the \textit{gft1} mutation likely represents a recessive allele of \textit{hom3}, which as mentioned above, encodes the aspartokinase. Evidence supporting this conclusion includes that all genomic clones from a wild type plasmid library that rescued growth of the \textit{gft1} mutant on EB-gal contained the full \textit{HOM3} open reading frame. I confirmed this result myself by showing that one such complementing genomic clone (pIS297) is able to rescue the growth defect phenotype of a \textit{gal3 hom3} yeast strain (Figure 8). Additionally, expression of a \textit{HOM3} ORF from a heterologous promoter also complements the \textit{gft1} defect, as does a Hom3-3X-FLAG-6His fusion vector, although to a lesser extent (Figure 8). Further confirmation was done to show that \textit{gal3 hom3} yeast show a \textit{gft} phenotype. Similar to the original \textit{gft1} mutant, \textit{gal3 hom3} yeast are unable to grow on EB-Gal (Figure 9A). I also found that the \textit{GAL1-GFP} reporter produced a drastically decreased response
in a gal3 hom3 strains and GAL for GFP expression compared to the gal3 strain (Figure 9B). In contrast, disruption of hom3 in a wild type GAL3 strain has little effect on induction of this reporter. Similar results were previously observed using a GAL-LacZ reporter gene where expression was monitored by expression of β-galactosidase (not shown).

I also confirmed earlier in vitro kinase assay results that were performed with the gft collection, which indicated that the gft1 mutation did not have a direct effect on Cdk8 kinase activity. For this purpose, I performed an in vitro kinase assay using FLAG-tagged full length Cdk8 recovered from wild type, gal3, gal3 hom3 or tor1 yeast strains. As substrates for these assays I used GST as a negative control, GST fused to the RNA Polymerase II CTD as a positive control, and full length wild type Gal4 expressed and purified from insect cells. I found that Cdk8-FLAG kinase recovered from all 4 strains by immunoprecipitation was capable of phosphorylating both Gal4 and GST-CTD to a similar extent, indicating that Cdk8 activity itself is unaffected by either the hom3 or gal3 mutations, confirming the previous results with the gft1 mutation (Figure 10).

Since HOM3 encodes aspartokinase, the first enzyme in the pathway for conversion of aspartate to methionine and threonine, I wondered whether the effect of the hom3 or gft1 mutation on GAL expression might be due to amino acid auxotrophy or by accumulation of aspartate, the substrate for Hom3 protein. To address this, I created mutants of genes encoding two additional enzymes in this pathway, HOM2 and HOM6. Interestingly, I found that the hom2 and gal3 hom2 strains did not show a growth defect when assayed on EB-Gal plates (Figure 11A). Similarly, the hom6 and gal3 hom6 strains also showed no growth defects when grown on EB-Gal (Figure 11A). These results indicate that the gft phenotype observed with gal3 hom3 is specific to a function of the Hom3 protein, and is not caused by a general defect in this metabolic
pathway. I also wondered whether a defect in aspartate kinase may cause accumulation of aspartate, which might inhibit GAL induction in gal3 yeast because of a feedback mechanism. To test this, I examined growth of gal3 yeast on EB-Gal media supplemented with excess aspartate. In this experiment, it appeared that gal3 yeast were still able grow as efficiently on this media as when grown on EB-Gal alone (Figure 11B). Taken together these results indicate that the growth defect of gal3 hom3 yeast is likely due to loss of the Hom3 protein itself rather than an effect of auxotrophy, or accumulation of its substrate.

I also examined whether Hom3 catalytic activity was required for its role in GAL gene expression (Espinosa-Cantu, 2018). To examine this, I created a mutation of an aspartate residue that is conserved within kinase enzymes, D297A, which was predicted to produce a catalytically inactive Hom3 protein. For this experiment, I transformed a gal3 hom3 yeast strain with a vector control (pRS314), a plasmid expressing wild type HOM3 ORF (pIS584), the same plasmid expressing the putative kinase inactive Hom3 (D237A), and the original genomic clone complementing construct (pIS297) as a positive control. These were plated on SD lacking Trp, SD lacking Trp, Thr and Met, and EB-Gal plates. The lack of Trp was required to select for the plasmids, while the absence of threonine and methionine was to examine whether the hom3 strain transformed with the plasmids are auxotrophic for these amino acids. I found that the strain possessing the vector control plasmid and the plasmid expressing the kinase inactive mutant were both unable to grow on the SD-Trp Thr Met plates, which confirms that the D237A mutation inhibits Hom3 activity (Figure 12). These strains were also examined for growth on EB-Gal to determine whether the D237A Hom3 mutant was able to rescue the gfi1 phenotype. Interestingly, only strains possessing the genomic HOM3 fragment clone or expressing the HOM3 ORF from the TEF1 promoter were able to grow on EB-Gal. These results indicate that
Hom3 kinase activity, rather than merely the Hom3 protein itself is required for gal3 yeast to induce their GAL genes by Cdk8-dependent long-term adaptation (Figure 12).

As shown above, gft1 was one of several mutants found to be sensitive to sub-lethal concentrations of rapamycin. Several previous observations have implicated a role for Hom3 in the Tor pathway as it was found to functionally interact with FKBP12 (Alarcon 1997), and hom3 deletion strains were observed to be rapamycin sensitive in a screen of the yeast haploid deletion strain set (Xie et al., 2005). Rapamycin sensitivity was confirmed in the W303 background using a gal3 hom3 strain (Figure 13). Both the hom3 and gal3 hom3 strains showed sensitivity on sublethal concentrations of rapamycin when compared to the wild type, and gal3 strains. I also examined whether there was a defect in Tor pathway signalling in the gal3 hom3 mutant by monitoring subcellular localization of the transcription factor Gat1. Gat1 is a well-characterized GATA transcription factor known to be regulated downstream of Tor signalling by subcellular localization; nutrient starvation or defects in Tor signalling cause nuclear localization of Gat1 (Georis, 2011). To examine this, wild type W303 yeast, tor1, and hom3 deletion strains were transformed with a construct expressing Gat1 fused to GFP, and the localization of Gat1 was examined by fluorescent microscopy. A Cdk8-GFP expression construct was also used as a positive control for nuclear localization. In wild type cells, Gat1 appears in the cytosol in cells growing on rich media as expected, but in both the tor1 and hom3 strains Gat1 appears to be predominately localized to the nucleus (Figure 14). These results confirm that the hom3 deletion must be causing a defect in Tor signalling.

Recently a novel assay was developed to measure TORC1 kinase activity in vitro (Tanigawa et al., 2017). Importantly, using this assay they were able to show sensitivity of the TORC1 complex to treatment with rapamycin (Tanigawa 2017). Because of the accumulating
evidence linking hom3 to the Tor pathway, I decided to examine whether the hom3 deletion has a
direct affect on TORC1 activity (Tanigawa 2017). Consequently, I performed this assay using
wild type, gal3, and hom3 strains, where the read-out for TORC1 activity is phosphorylation of
4-EBP1 (Figure 15). Consistent with the previously published report I found that rapamycin
treatment on wild type cells inhibited TORC1 activity in this assay (lane 4 and 5), which
confirms that the assay is capable of detecting inhibitory effects on activity. However, the
amount of 4-EBP1 phosphorylation produced by the TORC1 complexes from all three strains
appeared to be similar in this assay, indicating that the hom3 deletion does not affect TORC1
activity. A caveat of this experiment is that the TORC1 complex can utilize either Tor1 or Tor2,
and consequently it is possible that an effect of the hom3 deletion on Tor1 activity may be
obscured by redundancy for phosphorylation of 4EBP1 in this assay. Nevertheless, these results
indicate that the hom3 deletion mutant does not globally affect TORC1 activity, but rather may
inhibit signalling upstream or downstream of this complex.

The observation that hom3 and the gft1 mutants produce sensitivity to rapamycin, and
disruption of HOM3 causes constitutive nuclear localization of Gat1 indicates a role for this
protein in regulation of Tor signalling. Consequently, because Tor signalling seems to have
multiple upstream regulators and downstream target pathways, I examined several additional
features of the relationship between Hom3 and the Tor pathway for GAL expression in more
detail. Since there has been previous evidence that the Tor protein Fpr1 (Fkbp12) interacts with
Hom3 (Alarcon, 1997) I examined whether deletion of the fpr1 gene might modify the effect of
hom3 mutations on GAL expression. W303 strains bearing fpr1, gal3 fpr1, and gal3 hom3 fpr1
deletions were constructed and examined for growth on EB-Gal (Figure 16B). Both the fpr1, and
gal3 fpr1 were able to grow on EB-Gal, and gal3 fpr1 produced the “spotty” growth phenotype
typical of a gal3 background strain. Additionally, the gal3 hom3 fpr1 strain showed no rescue of the gft phenotype, and this strain grew on EB-galactose with identical properties as the strain with the gal3 deletion alone. These results indicate that the fpr1 deletion neither produces the gft phenotype on its own, nor does it suppress the effect of the hom3 deletion for GAL expression.

Tor signalling is known to regulate the activity of multiple downstream protein phosphatases, one of which is encoded by SIT4. Consequently, similar to the experiment described above, I examined whether deletions of sit4 might modify GAL induction in gal3 or hom3 gal3 yeast strains (Figure 16A). Again, I found that deletion of sit4 in an otherwise wild type background or in combination with gal3 produced strains that were still able to grow on EB-gal, and furthermore a triple gal3 hom3 sit4 strain produced an identical phenotype as the gft1/hom3 mutant. These results indicate that the Sit4 phosphatase, downstream of Tor is likely not involved in regulation of GAL gene expression.

As mentioned above, a separate pathway downstream of Tor is known to involve PP2A, comprised of the Pph1/2, Tpd3 and Cdc55 subunits. Consequently, I also examined whether mutations of CDC55 might affect the gft phenotype or regulation of GAL expression. Accordingly, I examined growth of W303 strains bearing gal3, gal3 hom3 and gal3 hom3 cdc55 deletions on EB-Gal (Figure 17). Interestingly, I found that deletion of cdc55 in the gal3 hom3 background shows a rescue of the gft phenotype and produces growth on EB-galactose similar to the gal3 strain. However, deletion of cdc55 in the wild type W303 background on its own does not affect growth on EB-gal. This indicates that disruption of CDC55 rescues the ability of gal3 hom3 yeast to induce their GAL genes, which suggests that PP2A phosphatase activity might counteract regulation of Gal4 by the long-term adaptation mechanism through Cdk8.
3.5 Mutation of Additional Tor Pathway Component Genes Produce the gft phenotype.

Since additional strains within the gft collection were found to be hypersensitive to rapamycin I decided to examine whether previously known components of the Tor pathway might be represented by one or more of these additional gft mutants. I began this analysis with the TORC1 complex, where the only non-essential members are Tor1 and Tco89 (Loewith, 2011). I first explored Tco89 by making tco89, and gal3 tco89 deletion strains and examining their ability to grow on EB-Gal (Figure 18A). In this experiment, I found that the tco89 strain was able to induce the GAL genes and grow on EB-gal as efficiently as wild type W303, but in contrast the gal3 tco89 strain was unable to grow, which mimics the gft mutant phenotype. Next, I examined whether disrupting CDC55 in combination with tco89 would rescue the gft phenotype as it did with the hom3 deletion. Here I found that the gal3 tco89 cdc55 strain was also able to grow on EB-Gal, similar to the strain with the gal3 deletion on its own, as was previously observed with the gal3 hom3 cdc55 strain. I also transformed the GAL1-GFP reporter into the tco89, and gal3 tco89 strains and GAL induction was monitored by GFP expression (Figure 18B). In these experiments, I found that strains expressing wild type Gal3 are able to induce the GAL genes regardless of whether Tco89 is present. However, induction decreased dramatically in gal3 tco89 cells, which is similar to the effect in gal3 hom3 cells and is consistent with the effect of these mutations for growth on EB-Gal.

I then looked at the phenotype of a tor1 deletion on the induction of GAL genes. W303 strains bearing tor1, and gal3 tor1 deletions were examined for growth on EB-Gal. While the otherwise wildtype strain with the tor1 deletion grew similarly to the W303 parent strain, the gal3 tor1 deletion mutant was unable to grow, indicating a defect in the ability to induce the GAL genes, exactly like a gft mutant (Figure 19A). Furthermore, a gal3 tor1 cdc55 triple deletion
strain was also found to grow on EB-gal, similar to the gal3 strain, which is consistent with the results shown above that inactivation of the Cdc55-PP2A phosphatase is able to rescue the gft phenotype. As previously, the GAL1-GFP reporter was also transformed into the tor1, and gal3 tor1 strains, and induction was monitored by GFP expression (Figure 19B). Again, consistent with the results shown above, both strains expressing wild type Gal3 were able to induce GAL expression independent of Tor1, whereas strains lacking gal3 and tor1 in combination were unable to induce expression of the GAL reporter, consistent with the phenotypes observed by growth on EB-gal plates.

After finding that mutants of TCO89 and TOR1 show a gft phenotype, I wanted to determine whether any of the original gft mutants represented mutations in these genes. For this analysis, I first mated the gal3 tco89 strain with each of the original gft mutants, and then examined growth of the diploids on EB-Gal plates. This was performed to determine if any of the gft mutants were non-complementing with the tco89 mutant for the gft phenotype which would indicate mutations in the same gene. For this analysis diploids from the cross were spotted on EB-Gal plates and growth was examined (Figure 20). From this it appeared that the gft7 mutant did not complement the tco89 deletion to the extent of the other mutants, indicating that gft7 may potentially represent an allele of tco89. Consequently, I examined this further by tetrad analysis to determine if the gft7 and tco89 mutants segregate separately. The diploid gft7/gal3 tco89 strain was sporulated, tetrad spores isolated and examined for growth on EB-Gal (Figure 21). As expected all 4 spores from this diploid were incapable of inducing their GAL genes as observed by the inability to grow on EB-Gal. In contrast, a control diploid was also used where an otherwise wild type gal3 strain was mated to the gal3 tco89 strain, and as expected, only 2 of
the 4 spores were unable to grow on EB-gal, typical of the gft phenotype. This analysis further confirmed that the gft7 mutation likely represents a recessive allele of tco89.

The same strategy was used for the tor1 deletion to determine whether one of the gft mutants may represent a mutation in tor1. For this analysis I mated the gal3 tor1 strain with each of the gft mutants, and then examined growth of the diploids on EB-Gal plates (Figure 22). From this experiment it appeared that gft6 did not fully complement the tor1 deletion mutant, and gft2 also appeared to only partially complement, and consequently I characterized these diploids further by tetrad analysis. The gft6/gal3 tor1, and gft2/gal3 tor1 diploids were sporulated, and the spores from tetrad analysis were examined for growth on EB-Gal (Figure 23). A control diploid was included where an otherwise wild type gal3 strain was mated to the gal3 tor1 strain. Surprisingly, 1 out of the 4 tetrads from both matings were capable of growth on EB-Gal, indicating that gft2 and gft6 are likely not allelic to tor1. Additionally, because the gal3 tor1 strain showed a gft phenotype I decided to more carefully examine the potential relationship with two additional mutants, gft5, and gft14 that showed hypersensitivity to rapamycin, which is also a phenotype of strains with disruption of tor1. However, for the gft5/gal3 tor1 diploid, I consistently obtained 3 out of 4 viable spores, indicating that there may be a synthetic lethal effect occurring during meiosis. For the gft14/gal3 tor1 diploid I found that 1 out of 4 spores grew on EB-gal plates, indicating that gft14 does not represent a tor1 mutant. Despite that the gal3 tor1 strain produces a gft phenotype, it appears that none of the gft collection are likely to represent mutations of tor1. This indicates that the original screen for gft mutants, defective in Cdk8-dependent induction of the GAL genes, must not have been completely saturating.
3.6 Tor-PP2A-Cdc55-dependent signalling opposes the effect of Cdk8-dependent Gal4 phosphorylation for GAL gene induction.

The parallel mechanisms for induction of the GAL genes are represented by Gal3 and relief of inhibition by Gal80, and Cdk8 phosphorylation of Gal4 (Rhode 2000). These 2 mechanisms were the rationale for the genetic screen using a gal3 background to isolate mutants defective in Cdk8 mediated induction of GAL expression. Cdk8-dependent induction of the GAL genes, as discussed above, is known to require phosphorylation of Gal4 at serine 699 by Cdk8, which allows induction by an as yet undefined mechanism but may involve destabilization of the Gal4-Gal80 interaction. Since cdk8/srb10 or Gal4 S699A mutations prevent the long-term adaptation effect in gal3 yeast, we reasoned that the gft mutants must somehow prevent phosphorylation at this site causing a lack of induction. Because disruption of the gene encoding the phosphatase PP2A subunit Cdc55 caused a rescue of the gft phenotype I wondered whether this phosphatase might become dysregulated because of defects in Tor signalling, as a consequence of the hom3/ gft1, tco89 and tor1 mutations such that it constitutively dephosphorylates Gal4 S699 and competes with Cdk8 for induction by this phosphorylation. To examine this hypothesis, I used a strain bearing deletions of both gal3 and cdk8/srb10 which is unable to induce the GAL genes at all, because both parallel mechanisms are non-functional, for disruption of CDC55. The strains were examined for growth on EB-Gal, and I found that both the gal3 srb10 and gal3 srb10 cdc55 were unable to grow on EB-Gal (Figure 24A), in contrast to that observed above for the effect of the cdc55 deletion for the gal3 hom3, gal3 tco89 and gal3 tor1 strains. This observation supports the hypothesis that Cdc55 must be affecting GAL induction by altering Cdk8-dependent modification of Gal4. In contrast, if the phosphatase was altering some other aspect of GAL gene induction, I expected that the cdc55 mutant would rescue
the \textit{gal3 srb10} phenotype. Further support for this hypothesis involved use of the Gal4 S699A mutant, which is epistatic to a \textit{cdk8} mutant for the \textit{gal3 gal} phenotype (Rohde, 2000). For this experiment I used strains bearing \textit{gal3} and \textit{gal4}, or \textit{gal3 gal4} and \textit{cdc55} and transformed them with plasmids expressing wild type Gal4 or a S699A mutant form of Gal4 (Figure 24B). I found that the \textit{gal3 gal4} strain expressing wild type Gal4 grew normally compared to otherwise wild type \textit{gal3} W303, as shown previously, but the same strain expressing the Gal4 S699A mutant was unable to grow on EB-Gal, similar to the \textit{gal3 srb10} mutant. Interestingly, while the \textit{gal3 gal4 cdc55} strain expressing wild type Gal4 grew on EB-gal similar to the \textit{gal3} W3030 strain, the same strain expressing the S699A Gal4 mutant was also unable to grow. This demonstrates that rescue of the \textit{gft} phenotype by disruption of \textit{cdc55} specifically requires phosphorylation of Gal4 at S699 by Cdk8. This supports the view that in the \textit{hom3}, \textit{tco89} and \textit{tor1} mutants PP2A-Cdc55 phosphatase activity may become dysregulated to cause constitutive dephosphorylation of Gal4 at serine 699 resulting in a lack of \textit{GAL} gene induction. This may explain why induction is rescued when \textit{cdc55} is disrupted, allowing Cdk8-dependent phosphorylation on Gal4 to accumulate, resulting in induction.
Figure 3. The *gft* screen: A genetic screen to isolate genes involved in Cdk8-dependent *GAL* induction.
Table 5: List of gft mutant phenotypes

<table>
<thead>
<tr>
<th>Strain</th>
<th>gft allele</th>
<th>Growth on EB-Gal</th>
<th>Cdk8 Activity</th>
<th>Invasive Growth</th>
<th>Growth on Rapamycin</th>
<th>Growth on MMS</th>
<th>Growth on HU</th>
<th>Growth on Hygromycin B</th>
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Figure 4. A GAL1-GFP reporter shows sensitive induction of the GAL genes in wild type strains. Yeast strain W303-1A (WT) was grown overnight in liquid selective media containing glycerol, lactic acid and ethanol as the carbon sources. Prior to induction a 1mL sample was taken and GFP expression measured by flow cytometry (NC). Galactose was added to final concentration of 2% from stock, and 1mL samples were taken 1, 2, 3, and 4 hours post induction and analysed by flow cytometry.
Figure 5. The GALI-GFP reporter shows that strains lacking gal3 display a long-term adaptation phenotype. Yeast strain ISY54 (gal3) was grown overnight in liquid selective media containing glycerol, lactic acid and ethanol as the carbon sources. Prior to induction a 1mL sample was taken and GFP expression measured (Neg. Control). Galactose was added to final concentration of 2% from stock, and 1 mL samples were taken 24, 48, 72, and 96 hours post induction.
Figure 6. *gal3* show sparse growth on EB-gal typical of the long-term adaptation phenotype. Yeast strains W303-1A (WT) and ISY54 (*gal3*) were grown overnight in liquid YPD. The OD A600 of all cultures were normalized and 1:100,000 dilutions were made for each strain. Then 200 μL were plated onto YPD or EB-galactose plates and incubated at 30°C for 5 days before being imaged.
Figure 7. The gft13-2 mutant appears to be allelic to a mutation in med2. A. Yeast strains W303-1A (WT), ISY54 (gal3), ISY66 (gal3med1), ISY58 (gal3med2), and ISY67 (gal3med9) were grown overnight in liquid YPD. The OD A600 of all cultures were normalized to 1.0. Then 10-fold serial dilutions were made and strains were spotted onto YPD or EB-galactose plates and incubated at 30°C for 5 days before being imaged. B. Yeast strain ISY58 (gal3med2) was mated to ISY54(gal3), ISY (gft3), or ISY(gft13-2), each diploid was sporulated and tetrads dissected as described in Materials and Methods. Germinated spores from each respective cross was grown overnight in liquid YPD, normalized to an OD A600 of 1.0 and 10-fold serial dilutions were made. Dilutions were spotted on YPD or EB-galactose plates, grown for 5 days at 30°C and photographed.
Figure 8. The *gft1* phenotype is complemented by a plasmid expressing *HOM3*. Yeast strains ISY54(*gal3*) and ISY128(*gal3*<sup>hom3</sup>) were transformed with the control plasmid pRS314, pIS297 (genomic *HOM3*) or pIS556 (*HOM3*-FLAG). Transformants were grown overnight in selective liquid media, normalized to an OD A600 of 1.0 and 2-fold serial dilutions were made. Dilutions were spotted on EB-galactose plates, grown for 5 days at 30°C and photographed.
Figure 9. Confirmation that \textit{gal3 hom3} strains produce the \textit{gft1} phenotype for \textit{GAL} induction. A. Yeast strains W303-1A (WT), ISY54 (\textit{gal3}), ISY279 (\textit{hom3}), ISY128 (\textit{gal3hom3}), and ISY67 (\textit{gft1-1}), bearing a \textit{GAL1-GFP} reporter gene were grown overnight in liquid YPD. The OD A600 of the cultures were normalized to 1.0, then 10-fold serial dilutions were made and spotted onto YPD or EB-galactose plates before being incubated at 30°C for 5 days and imaged. B. Yeast strains W303-1A (WT) (left ○), ISY279 (\textit{hom3}) (left □), ISY54 (\textit{gal3}) (right ○), and ISY128 (\textit{gal3hom3}) (right □) were grown in triplicate overnight in liquid selective media containing glycerol, lactic acid and ethanol as the carbon sources. Prior to induction a 1mL sample was taken and GFP expression measured by flow cytometry (Time = 0). Galactose was added to final concentration of 2% from a stock, and 1 mL samples were taken at the specified times post induction.
Figure 10. Cdk8 activity in vitro is not altered in the gft1 mutant. Yeast strains W303-1A (WT), ISY54 (gal3), ISY128 (gal3 hom3), and yNH008 (tor1) were transformed with pIS484 expressing full length wild type Cdk8-FLAG tagged. In vitro kinase reactions were performed with either 10 μL purified GST (lanes 1, 4, 7, 10), 2 μL GST fused RNAPII CTD (lanes 2, 5, 8, 11) or 2 μL purified recombinant Gal4 protein (lanes 3, 6, 9, 12) as the substrates.
Figure 11. The gft1 phenotype is specific to hom3 in the aspartate metabolic pathway. A. Yeast strains W303-1A (WT), ISY54 (gal3), yNH014 (hom6), yNH015 (gal3 hom6), ISY311 (hom2), and ISY277 (gal3 hom2) were grown overnight in liquid YPD. The OD A600 of all cultures were normalized to 1.0, then 10-fold serial dilutions were made and spotted onto YPD or EB-galactose plates before being incubated at 30°C for 5 days and imaged B. Strains W303-1A (WT) and ISY54 (gal3) were grown and diluted as above. Dilutions were spotted on YPD, EB-galactose or EB-galactose with 5mM excess aspartate added and incubated at 30°C for 5 days then photographed.
Figure 12. Kinase activity of Hom3 is required for induction of the GAL genes. The yeast strain ISY128 (gal3hom3) was transformed with plasmids pRS314 (vector control), pIS574 (HOM3 ORF), two clones of pNH01, expressing the Hom3 the D237A mutation, which is predicted to inactivate kinase function, and pIS297 bearing a genomic clone of HOM3 as a positive control. Transformants were grown overnight in selective media with 2% glucose, diluted to an OD A600 of 1.0, and serially diluted 10-fold. Dilutions were spotted on SC-Trp, SC-Trp-Met-Thr, and EB-galactose, and grown for 3 days on SC media and 5 days on EB-galactose at 30°C prior to being imaged.
Figure 13. The gft1/hom3 mutant is hypersensitive to sub-lethal concentrations of rapamycin. Yeast strains W303-1A (WT), ISY54 (gal3), ISY279 (hom3), and ISY128 (gal3 hom3) were grown overnight in liquid YPD. The OD A600 of all cultures were normalized to 1.0, then 10-fold serial dilutions were made and spotted onto YPD or YPD containing 5 ng/mL rapamycin plates before being incubated at 30°C for 3 days and imaged.
Figure 14. The *hom3* mutant causes constitutive nuclear localization of the Tor pathway target Gat1. Yeast strains W303-1A (WT), yNH008 (*tor1*), and ISY279 (*hom3*) were transformed with pRS416GAT (Gat1-GFP), and WT was also transformed with pJP015 (Cdk8-GFP). Cells were prepared as described in Material and Methods and visualized using fluorescent microscopy. DNA was visualized using Hoechst stain.
Figure 15. The *hom3* mutant does not directly affect TORC1 activity. TORC1 activity was assessed in semi-permeable yeast cells made from strains W303-1A (WT) (lane 1), ISY54 (*gal3*) (lane 2), and ISY279 (*hom3*) (lane 3). Rapamycin, inhibitor of TORC1, was included as a negative control at 1 μM (lane 4) and 10 μM (lane 5). The *in vitro* kinase assays were run for 10 minutes at 30°C. The samples were western blotted for total 4EBP1 and phosphorylated 4EBP1.
Figure 16. Mutations in SIT4 and FPRI do not affect the gft phenotype. A. Yeast strains W303-1A (WT), ISY54 (gal3), ISY128 (gal3 hom3), ISY281 (gal3 sit4), and ISY282 (gal3 hom3 sit4) were grown overnight in liquid YPD. The OD A600 of all cultures were normalized to 1.0, then 10-fold serial dilutions were made and spotted onto YPD or EB-galactose plates before being incubated at 30°C for 5 days and imaged B. Strains W303-1A (WT), ISY54 (gal3), ISY128 (gal3 hom3), ISY136 (fpr1), ISY137 (gal3 hom3 fpr1), and ISY138 (gal3 fpr1) were grown and plated as described above.
Figure 17. The additional disruption of \textit{cdc55} rescues the \textit{hom3 gft} phenotype. Yeast strains W303-1A (WT), ISY54 (\textit{gal3}), ISY279 (\textit{hom3}), ISY128 (\textit{gal3 hom3}), and yNH011 (\textit{gal3 hom3 cdc55}) were grown overnight in liquid YPD. The OD A600 of all cultures were normalized to 1.0, then 10-fold serial dilutions were made and spotted onto YPD or EB-galactose plates before being incubated at 30°C for 5 days and imaged.
Figure 18. Disruption of tco89 produces the gft phenotype. A. Yeast strains W303-1A (WT), ISY54 (gal3), yNH016 (tco89), yNH017 (gal3 tco89), yNH025 (gal3 cdc55), and yNH028 (gal3 tco89 cdc55) were grown overnight in liquid YPD. The OD A600 of all cultures were normalized to 1.0, then 10-fold serial dilutions were made and spotted onto YPD or EB-galactose plates before being incubated at 30°C for 5 days and imaged. B. Yeast strains with the GAL1-GFP reporter yNH029 (WT) (left ○), yNH035 (tco89) (left □), yNH030 (gal3) (right ○), and yNH036 (gal3 tco89) (right □) were grown in triplicate overnight in liquid selective media containing glycerol, lactic acid and ethanol as the carbon sources. Prior to induction a 1mL sample was taken and GFP expression measured (Time = 0). Galactose was added to final concentration of 2% from a stock, and 1 mL samples were taken at the specified time points post induction. GAL expression is indicated as an average of the mean fluorescent intensities of the cultures.
**Figure 19. Deletion of tor1 produces the gft phenotype in gal3 yeast.** A. Yeast strains W303-1A (WT), ISY54 (gal3), yNH008 (tor1), yNH009 (gal3 tor1), and yNH019 (gal3 tor1 cdc55) were grown overnight in liquid YPD. The OD A600 of all cultures were normalized to 1.0, then 10-fold serial dilutions were made and spotted onto YPD or EB-galactose plates before being incubated at 30°C for 5 days and imaged. B. Yeast strains with the GAL1-GFP reporter yNH029 (WT) (left ○), yNH033 (tor1) (left □), yNH030 (gal3) (right ○), and yNH034 (gal3 tor1) (right □) were grown in triplicate overnight in liquid selective media containing glycerol, lactic acid and ethanol as the carbon sources. Prior to induction a 1 mL sample was taken and GFP expression measured (Time = 0). Galactose was added to final concentration of 2% from a stock, and 1 mL samples were taken at the specified time points post induction. GAL expression is indicated as an average of the mean fluorescent intensities of the cultures.
Figure 20. Diploids produced from crosses of the gft mutant collection indicates that tco89 may be allelic with gft7. The yeast strain yNH017 (gal3 tco89) was mated to either ISY54 (gal3) or each member of the gft collection (ISY158-ISY171). The diploids were then grown overnight in selective media, the OD A600 of all cultures were normalized to 1.0, then 10-fold serial dilutions were made and spotted onto YPD or EB-galactose plates before being incubated at 30°C for 5 days and imaged.
Figure 21. Tetrad analysis confirms that \textit{tco89} is allelic with \textit{gft7}. Yeast strain yNH017 (\textit{gal3} tco89) was mated to ISY54 (\textit{gal3}) or ISY192 (\textit{gft7}) and each diploid was sporulated and tetrads dissected as described in Materials and Methods. Each spore from the diploids was grown overnight in liquid YPD, normalized to an OD A600 of 1.0 and 10-fold serial dilutions were made. Dilutions were spotted on YPD or EB-galactose plates, grown for 5 days at 30°C and photographed.
Figure 22. Complementation analysis of a tor1 mutation with the gft mutant collection. The yeast strain yNH009 (gal3 tor1) was mated to either ISY54 (gal3) or each member of the gft collection. The diploids were then grown overnight in selective media, the OD A600 of all cultures were normalized to 1.0, then 10-fold serial dilutions were made and spotted onto YPD or EB-galactose plates before being incubated at 30°C for 5 days and imaged.
Figure 23. Tetrad analysis indicates that tor1 is likely not allelic with gft2, gft6, or gft14. Yeast strain yNH009 (gal3 tor1) was mated to ISY54 (gal3) (positive control), ISY187 (gft2), ISY191 (gft6), or ISY200 (gft14), then each diploid was sporulated and tetrads dissected as described in Materials and Methods. Each spore from the diploids was grown overnight in liquid YPD, normalized to an OD A600 of 1.0 and 10-fold serial dilutions were made. Dilutions were spotted on YPD or EB-galactose plates, grown for 5 days at 30°C and photographed.
Figure 24. Cdc55 affects GAL induction by a mechanism involving Cdk8/Srb10 and phosphorylation of Gal4 S699. A. Yeast strains W303-1A (WT), ISY54 (gal3), yNH003 (srb10), yNH024 (gal3 srb10) and yNH026 (gal3 srb10 cdc55) were grown overnight in liquid YPD, normalized to an OD A600 of 1.0 and 10-fold serial dilutions were made. Dilutions were spotted on YPD or EB-galactose plates, grown for 5 days at 30°C and photographed. B. Yeast strains YJR40 (gal3 gal4) and yNH037(gal3 gal4 cdc55) were transformed with plasmid YCpG4trp (WT Gal4) or pRD038 (S699A Gal4). The strains were grown overnight in liquid SC-trp, normalized to an OD A600 of 1.0 and 10-fold serial dilutions were made. Dilutions were spotted on SC-Trp or EB-galactose plates, grown for 5 days at 30°C and photographed.
CHAPTER 4: DISCUSSION

4.1 Summary of Findings

The GAL gene regulon in *S. cerevisiae* is an excellent model for the study of eukaryotic gene regulation. A genetic screen was performed utilizing this model to uncover upstream regulators of Cdk8 by exploiting the Cdk8-dependent pathway of induction, which successfully identified 16 *gft* mutants which prevent Cdk8-dependent GAL expression. The identity of most of these mutants remains unknown, so I examined growth of these mutants under various conditions to identify additional associated phenotypes. This revealed that one of the key signalling pathways in yeast represented by the target of rapamycin, may be involved, as shown by the rapamycin sensitivity displayed by some of the mutants. Interestingly, only a fraction of the *gft* mutants showed altered Cdk8 kinase activity when the enzyme was assayed *in vitro*. My thesis research focused mainly on the mutants of this class that do not appear to have a direct effect on Cdk8 activity. One mutant, *gft1*, was found to represent a recessive allele of *hom3*, which encodes a metabolic aspartokinase in the biosynthetic pathway for threonine and methionine. Characterization of *gft1* revealed a defect in Tor signalling; strains defective for *gft1/hom3* are hyper-sensitive to rapamycin and cause constitutive Gat1 nuclear localization. It was also found that the kinase activity of Hom3 is necessary to suppress the *gft* phenotype, as shown by the inability of *gal3 hom3* yeast transformed with a Hom3 bearing a D237A mutation, which alters a conserved residue in the kinase domain, to grow on EB-Gal. Furthermore, null mutations of *tor1* or *tco89*, encoding components of the TORC1 complex, also prevent GAL expression in *gal3* yeast. Through tetrad analysis it was revealed that *gft7* is likely allelic to a *tco89* mutant. Despite that *tor1* mutants display a *gft* phenotype, tetrad analysis with diploids produced with the *gft*
mutants, indicates that tor1 is likely not represented within the initial collection of gft mutants. Further genetic analysis demonstrated that disruption of cdc55, encoding a regulatory subunit of the PP2A protein phosphatase downstream of Tor signalling suppresses the effect of hom3, tco89, and tor1 mutations on GAL expression.

Members of the mediator complex were also examined as potential gft mutants as this might provide a mechanistic explanation for the gft phenotype that is separate from the Tor pathway. It was found that med2 gal3 yeast were unable to utilize galactose when grown on EB-Gal. Screening the gft collection for sensitivity to sub-lethal concentrations of hygromycin B, which was previously shown to represent a phenotype for med2 mutants, revealed four potential gft mutant candidates. These were mated to gal3 med2, sporulated and tetrad analysis was performed. This uncovered that med2 is allelic to gft13-2.

4.2 Hom3 as a gft mutant and its role in Tor signalling

Much of the work in this thesis focuses on characterizing gft1, which was found to represent a recessive allele of hom3. Despite confirming that the gft phenotype observed with the gal3 hom3 mutant was specific to Hom3, since other enzymes in the aspartate metabolic pathway, including Hom2 and Hom6 did not show this phenotype. The hyper-sensitivity to rapamycin appears to be caused by mutations of all members of this pathway (Xie et al., 2005). This indicates that the role Hom3 plays in the Tor pathway may be dependent on the aspartate metabolic pathway and is likely linked to amino acid metabolism. However, the unique gft phenotype shown only by the hom3 mutant suggests that the Hom3 enzyme has an additional, more elaborate regulatory role. This is supported by the rescue of this gft phenotype in the gal3 hom3 cdc55 mutant strain, leading to the possibility that Hom3 under wild type conditions aids in the regulation of this phosphatase complex downstream of Tor signalling. This would explain
why when hom3 is mutated PP2A-Cdc55 may become dysregulated leading to inhibited GAL gene induction by a mechanism involving dephosphorylation of Gal4 at serine 699, which produces the gft phenotype.

I considered the possibility that Hom3 protein may have a moonlighting function, where it acts in another role that is separate from its aspartokinase activity. However, I found that kinase activity of the enzyme was required to suppress the effect of the hom3l gft1 mutation which implies that aspartokinase catalytic function is required for its role in GAL induction, rather than simply the protein itself. A caveat to this interpretation is that because antibodies against Hom3 protein are not available, I was not able to confirm that the D237A mutant protein was actually expressed, and furthermore a C-terminal 3X-FLAG-6his Hom3 fusion was found to be incapable of complete complementation of the gft1 mutation.

Hom3 seems to be somehow involved in regulation of Tor signalling, given that hom3 null mutations cause rapamycin sensitivity, and cause constitutive nuclear localization of the downstream target factor Gat1. However, precisely how hom3 mutants affect Tor signalling remains an enigma. I attempted to determine whether Hom3 was required for TORC1 activity using a recently described in vitro kinase assay (Tanigawa et al., 2017), but it appeared that the hom3 mutation had no effect in this assay. A complication of this interpretation is that TORC1 activity can involve either the Tor1 or Tor2 isoform and Tor2 is known to be redundant to Tor1 for most of its functions (Loewith and Hall, 2011). This means that even if hom3 mutations affect Tor1 activity specifically, the presence of Tor2 in the TORC1 would likely mask this effect making it appear as though TORC1 activity was unchanged. Interestingly however, with the original description of the in vitro TORC1 kinase assay, of various amino acids that were added to the reaction only aspartate was shown to have an inhibitory effect (Tanigawa et al.,
2017). Consequently, a simplistic hypothesis might be that the Tor signalling defect is produced by accumulation of intracellular aspartate because of defective Hom3 function. However, I was unable to confirm this possibility in that addition of high concentrations of aspartate to the growth media did not produce even a small effect on growth of gal3 yeast on EB-gal plates. An additional observation possibly linking Hom3 to Tor signalling is that this protein was discovered by high-throughput affinity capture MS proteomics studies in complexes with both Cdc55 and Fpr1 (Breitkreitz et al., 2010). The latter interaction with Fpr1, the receptor protein for rapamycin and a known regulator of Tor was also previously identified in a 2-hybrid screen, and in this study Fpr1 was shown to influence the function of aspartokinase (Alarcon and Heitman, 1997). However, my studies have shown that deletion of fpr1 does not produce a similar gft phenotype as hom3 deletions, nor does it suppress the effect of gft1/ hom3 mutations. Consequently, it does not seem likely the interaction between Hom3 and Fpr1 plays a role for Tor signalling, at least for induction of the GAL genes. A potential role for interaction of Hom3 with Cdc55 for Tor signalling seems attractive, considering that cdc55 mutants suppress effects of hom3, tor1 and tco89 deletions for the gft phenotype. However, I was unable to confirm this interaction in my studies because, as mentioned above antibodies against Hom3 are not yet available, and C-terminal 3X-FLAG-6his Hom3 fusion protein was unable to fully compliment the gft1 defect for GAL induction. Consequently, then, additional approaches such as production of antibodies against Hom3 will be required to determine how Hom3 function may regulate the Tor pathway.

4.3 Model for regulation of the GAL genes by Tor signalling

Despite that the precise role of Hom3 for regulation of Tor signalling remains undetermined, my results have clearly demonstrated a role for this nutrient responsive pathway
for regulation of $GAL$ gene expression. The current model for Cdk8-dependent induction of the $GAL$ genes, which produces the LTA phenotype in $gal3$ strains, is that the activator Gal4 is phosphorylated at serine 699 by Cdk8 and that this modification may alter the interaction between Gal4 and the inhibitor Gal80. In this view the $gft$ mutants are predicted to inhibit this Cdk8-dependent pathway, either by disrupting Cdk8 activity directly or by otherwise altering the phosphorylation state of Gal4 on serine 699 (Rohde et al., 2000). Since neither of the $gft$ mutants represented by $hom3$ and $tco89$ appear to alter Cdk8 activity, it seems more likely that these mutants must alter the phosphorylation of Gal4 by another mechanism \emph{in vivo}. This is supported by the observation that induction of the $GAL$ genes in these mutants can be rescued by disruptions of $cdc55$. From these observations, we propose that Cdk8 phosphorylation of serine 699 of Gal4 may be opposed by one or more protein phosphatases downstream of Tor. In this view, the set of Tor-related $gft$ mutants do not affect Cdk8 function directly, however disruption of $hom3$, $tor1$ or $tco89$ may cause hyperactivation of PP2A-Cdc55, which may prevent accumulation of Gal4 phosphorylation at serine 699 (Figure 25). Disruption of $cdc55$ may inactivate this phosphatase function, rendering it incapable to act on Gal4, which would allow phosphorylation on serine 699 to accumulate allowing induction of the $GAL$ genes. Similar competition between kinase and phosphatases have been previously described, one example relating to the Tor regulated type 2C phosphatases (Cheng et al., 1999; Gonzalez et al., 2009). Accordingly, it was found that the 2C phosphatases Ptc 2 and Ptc3 act on Cyclin-dependent kinases themselves (Cdk5) but also on many substrates of these kinases (Cheng et al., 1999; Gonzalez et al., 2009). A complication of the model proposed here (Figure 25) lies in whether Cdc55 normally acts on Gal4 or whether it becomes dysregulated and only uses Gal4 as a substrate when $hom3$, $tor1$ or $tco89$ are mutated. My results present genetic evidence for an
opposing relationship between Tor signalling to PP2A-Cdc55 function for phosphorylation of Gal4 at serine 699 by Cdk8. Confirmation of this model will require further biochemical analysis of both PP2A phosphatase function and the Gal4 phosphorylation state in the Tor signalling mutant backgrounds.

4.4 Med2 represents a separate but distinct group of the gft mutants

Although the majority of the gft mutants characterized in this work were involved in the Tor pathway in some aspect, additional phenotypes suggest that med2 represents a different class amongst the gft mutant collection. Non-essential genes encoding components of mediator were examined for their potential as gft mutants, for the obvious reason that they interact with the Cdk8-module. This analysis resulted in identification of med2 as a gft mutant, which is different from the Tor-related gfts because it is not hyper-sensitive to rapamycin and has no known established role in the Tor pathway. It was also found that gft13-2/med2 shows slightly altered Cdk8 activity as assayed in vitro. Since it is known that the middle module of mediator interacts with the Cdk8 module, the role of Med2, as a member of the tail module was unclear. However, there is strong evidence that the mediator tail module essentially functions as a positive regulator of mediator function overall and promotes the interaction between mediator and UAS-bound activators (Jeronimo et al., 2016). We do not observe complete loss of Cdk8 activity in the med2 mutant, which may be supported by the emerging view that the “core mediator” should be redefined as the minimal, essential and functional mediator complex which does not include the tail module (Jeronimo et al., 2016, Tsai et al., 2014). This means that the tail module may be dispensable for minimal mediator activity, and its presence acts to enhance the already existent basal activity (Jeronimo et al., 2016, Tsai et al., 2014). This may explain why med2 strains do not show complete loss of Cdk8 activity.
4.5 Significance of Findings

Dysregulated transcription is at the root of many diseases, including cancer, which is a leading cause of mortality in modern societies. This makes the study of transcriptional regulation key in understanding not only normal development but also tumorigenesis. As previously discussed, the mediator complex is an important regulator of the majority of RNAPII transcribed genes (Kim et al., 1994). The specific role of Cdk8 activity in this regulation appears to be context-specific and depend on the identity of transcription factors with which it uses as substrate (Xu and Ji, 2011). To date there are no known upstream regulators of Cdk8, in either yeast or mammalian cells, despite it playing a crucial role in transcriptional regulation. The gft mutant screen was designed to address this issue, and with further characterization of the collection, new regulators will be uncovered. The regulation of this enzyme has become of particular interest in recent years due to emerging evidence that the genes for Cdk8 and Cyclin C are either mutated or amplified in a variety of human cancers, specifically melanoma and colorectal (Kapoor et al., 2010; Xu and Ji, 2011). Cdk8 was identified as an oncoprotein that promotes the proliferation of colorectal cancer cells (Xu and Ji, 2011). In addition to amplification of Cdk8 in colorectal cancer, elevated expression of the CDK8 gene is reported to play a major role in promoting the proliferation of melanoma cells (Kapoor et al., 2010). Besides overexpression, a Cdk8 point mutation (D189N) was found in diverse tumor samples, but the functional consequence of this mutation has yet to be determined (Greenman et al., 2007). All of this evidence highlights the importance of properly regulated Cdk8 function, and how insight into the regulation of this enzyme may aid in better understanding human disease.
4.6 Future Directions

The work presented here has contributed to understanding proteins involved in Gal4 phosphorylation and dephosphorylation, which is important for GAL gene induction, however there are many questions that remain unanswered. An important immediate objective would be to provide biochemical demonstration showing altered Gal4 phosphorylation in gft1 and gft7 mutant strain backgrounds in vivo. The data presented in this thesis leads us to believe that the phosphorylation state of Gal4 must be altered in these gft mutants, and while phosphorylation should accumulate on Gal4 in strains with deletions of cdc55, however in my research, for technical and reagent limitations, I have had difficulty detecting phosphorylated Gal4 protein in vivo.

A larger portion of future work relating to this project will involve characterizing the 13 outstanding currently unidentified gft mutants. The approach I describe in this thesis was not systematic and relied primarily on educated guesses and perhaps a bit of luck. In the future I would hope to take a more systematic approach to identifying the remaining gft mutants. The previous work to identify hom3 as gft1 involved using complementation with a genomic library, but initial subsequent attempts to identify the remaining mutants of the gft collection, most of which produce more subtle phenotypes as measured by growth on EB-gal plates have been unsuccessful. I propose that it may be more productive to screen a library expressing defined ORFs. This may eliminate problems associated with screening of random genomic fragments, and hopefully systematically to identify the remaining mutants. Additionally and alternatively, it may be possible to identify at least some of the remaining gft mutants by genome re-sequencing. However, the success of this approach will likely require additional back crossing of most of the mutants, as initial genome re-sequencing of 6 mutant genomes, which had defects in Cdk8 kinase
activity, even though backcrossed 10 times, produced thousands of polymorphisms that significantly complicate identification of a relevant genetic alteration. In retrospect, these persistent polymorphisms may be due to the relatively subtle nature of the *gft* phenotype, which were produced by random mutagenesis using UV irradiation. Undoubtedly, identification of the additional mutants will reveal new information regarding Cdk8 function.
Figure 25. Summary of involvement of the TOR pathway in the regulation of the GAL genes. A schematic model of the TOR pathway and potential interaction with the GAL genes. Only enzymes relevant to this study are represented. Solid lines represent confirmed interactions and dotted lines are unconfirmed interactions.
REFERENCES


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