Chapter 4

To Study the regulation transcription factors involved in cell cycle and morphogenesis in *C. albicans* expressing ubiquitin mutations.

4.1. Introduction:

is a clinically noteworthy fungal pathogen capable of causing candidiasis and candidemia in humans affecting a wide range of organs. Understanding the molecular mechanism of its pathogenesis is crucial for developing effective therapeutic interventions. Ubiquitin-mediated protein degradation is a highly conserved mechanism that regulates various cellular processes in eukaryotes through the Ubiquitin proteasome system (UPS) pathway (Hochstrasser et al., 2009). The UPS tightly regulates the degradation of proteins that play important roles in various cellular pathways involving viability and host adaptation in fungi. Therefore, understanding the regulation of the UPS through ubiquitin mutants in fungal pathogenesis may be valuable for the future development of novel therapeutic approaches. Drug development against targets of the fungal UPS has not been explored extensively (Cao et al., 2021). In fungal pathogens, ubiquitin genes have been shown to play an important role in the development, stress resistance and fungal virulence (Villamón et al., 2004, Oh et al., 2012, Liu et al., 2018). Mutations in ubiquitin can disrupt protein homeostasis and cellular functions in Eukaryotes (Nakayama et al., 2006), potentially affecting the virulence of pathogens such as *C. albicans* (Dantuslia et al., 2023). While previous studies have highlighted the importance of ubiquitin in eukaryote biology, the specific effects of ubiquitin and ubiquitination on protein expression during morphogenesis and pathogenesis of *C. albicans* remain poorly understood. The role of ubiquitin in cellular processes extends beyond protein degradation, encompassing aspects of signaling, DNA repair, and cell cycle regulation (Kornitzer et al., 2000). In *C. albicans*, ubiquitin-mediated proteolysis has been implicated in morphological transitions, stress response, and virulence factor regulation (Hossain et al., 2011, Yang et al., 2020). By elucidating the roles of ubiquitin in biological response pathways and metabolic processes, present study aims to broaden the understanding of virulence of C. albicans and identify novel therapeutic targets to combat fungal infections. Here ubiquitin mutations were employed to unravel the role of ubiquitin regulation in the molecular mechanisms of pathogenesis of C. albicans. Proteomics approach offers a comprehensive view of cellular protein dynamics and has been instrumental in clarifying the effects of genetic perturbations on the proteome (Peng et al., 2003). Earlier studies from our laboratory pointed out that certain mutations of ubiquitin namely UbEP42, UbL50P, and UbI61T interfere with the cell cycle, antibiotic stress, thermotolerance, polyubiquitin chain formation, morphogenesis and virulence of *C. albicans* (Dantuslia et al., 2023). Hence, the same mutations were chosen here to

find the differences in protein profile to pinpoint the biological and metabolic changes that contribute to morphogenesis and virulence. The combination of genetic manipulation of ubiquitin and proteomics helped in understanding the influence of ubiquitin-dependent processes over cellular physiology in *C. albicans*.

4.2. Materials and Methods:

4.2.1. Strains and Media:

The auxotrophic avirulent strain of *Candida albicans*, BWP17, was used in the present studies (Wilson et al., 1999). BWP17 (*ura3*::*1 imm434/ura3*::*1 imm434 his1*::*hisG/his1*::*hisG arg4*::*hisG/arg4)*, a derivative of CAI4, was generously provided by Prof. Yue Wang from IMCB Singapore. BWP17 cells were cultured in YPD medium (1% w/v yeast extract, 2% w/v peptone, 2% w/v dextrose), with or without 50 μg/ml uridine for selection, as described previously [13]. For the proteomics approach, we selected only dosage dependent lethal ubiquitin mutants UbL50P, UbI61T, and UbEP42 out of the five mutants (UbS20F, UbA46S, UbL50P, UbI61T, and UbEP42) previously used for preliminary investigations involving *C. albicans* and pTET25MN-C vector was used to express the mutant genes of ubiquitin in BW17 strain previously described (Dantuslia et al., 2023). The transformants of wild-type ubiquitin UbWT were used as the positive control.

4.2.2. Analysis of the expression levels of virulence-associated transcription factors:

Quantitative real-time PCR was carried out for comparative analysis of the expression of virulence-associated transcription factors. The total RNA of the BWP17 transformants of the wild type and the mutant forms were isolated using TRIzol reagent (Takara, Japan). Poly-A tailing of small RNAs was performed using E. coli Poly-A Polymerase (New England Biolabs, UK) at 37 °C for 30 min. cDNA was synthesized using a cDNA prime script synthesis kit (Takara, Japan). Primers used for transcriptional analysis of transcription factor are listed in (Table 4.3) One-way analysis of variance (ANOVA) followed by Bonferroni's test was used for intergroup variation analysis. Statistical significance was established at $p < 0.05$.

4.2.3. Sample preparation for proteome analysis:

Proteome was extracted using the modified trichloroacetic acid (TCA)/acetone precipitation method, as described by (Peng et al. 2003). Briefly, cell pellets were lysed in lysis buffer

containing 8 M urea, 2 M thiourea, 4% CHAPS, 65 mM DTT, and 0.2% Bio-Lyte. The resulting precipitate was collected and washed three times with PBS before storing at -80°C. The concentration of proteins in the samples was determined using the Bradford assay, following the protocol outlined. Proteins were reduced with 10 mM DTT, alkylated with 55 mM iodoacetamide, and digested with 6 ng/ml trypsin (Sigma Sequencing Grade Modified) for 16 h at 37°C. The trypsin reaction was stopped by the addition of an equal volume of a 0.2% formic acid solution, and the peptides were extracted using 50% acetonitrile in 0.1% formic acid. Peptides were desalted using C18 spin columns. Subsequently, the peptides were analyzed using an LC-MS/MS system. During the MS analysis, proteins were ionized and peptides were separated on a C18 reverse-phase column using an acetonitrile gradient at a flow rate of 300 nL/min over a linear gradient of 90 min. The mass spectrometer was operated in data-dependent mode with positive polarity, utilizing an electrospray voltage of 2 kV. Full scan MS spectra (m/z 300–1600) were acquired in the orbitrap at a resolution of 70 K, with an automatic gain control (AGC) target of 1e6 and a maximum injection time of 80 ms. The top 10 intense ions were selected for HCD MS/MS fragmentation, with MS2 resolution set at 17500 and an AGC target of 2e5. Fragmentation was achieved using a normalized collision energy (NCE) of 32% and a dynamic exclusion duration of 15 s. MS data acquisition was performed in data-dependent mode, and subsequent raw file processing was carried out using MaxQuant software for protein identification and quantification, following the parameters specified (Peng et al.2003). These parameters included a precursor mass tolerance of 10 ppm, a fragment tolerance of 0.02 Da, dynamic modifications for oxidation (M), and a static modification for carbamidomethyl (C).

4.2.4. Data Analysis:

Differential expression analysis of proteins was performed using Perseus software (Gierlinski et al., 2018). Proteins with a fold change ≥ 1.5 and a p-value < 0.05 were considered differentially expressed. Identified proteins were subjected to bioinformatics analysis to elucidate their biological functions, subcellular localization and interactions. Gene ontology (GO) enrichment analysis and pathway analysis were performed to identify overrepresented biological processes and metabolic pathways. The consensus upregulated and downregulated genes were uploaded into the STRING database, and a high confidence interaction score ≥ 0.7 was used to reduce

false-positive interactions. The resultant network output was loaded into Cytoscape to obtain metabolic pathway information.

Table 4.3 Primers used for transcription factors analysis

RT pcr primer		
Efg1p	Forward	CCTGCCGCAACATCTCAAGG
	Reverse	TGCTGAGGTTGTGGCTGTGA
Nrg1p	Forward	GCCAACATGTGAAGCCCGTT
	Reverse	CCTGTGTTGTTGTCTGTTGCGT
Rbf1p	Forward	CAGCTGTTGCGTCTCAAGGA
	Reverse	AGCATCGTGCTCATTACCCTGT
Rim101p	Forward	GTACAACACACCAGCTTGACC
	Reverse	GTGGATTGACGCAGTTAGATGG
Stp1p	Forward	CAGGTGCATGCGCCATCTTC
	Reverse	GTGGAATGGCGGTGGTTGTC
Fkh2	Forward	CGAAAAAGCGTCCCCACTCAC
	Reverse	TTGGTTTTCTCCTCTGGAGCC
Tec1p	Forward	TCCCGCAGTTGCCAGAATCA
	Reverse	TGGTATGTGTGGGTGATGCGT
Cln3	Forward	CAGCAGTAACTTTGAGGTTG
	Reverse	GTCTTGGGTGATAATGGTGT
Hgc1	Forward	TAGTCAGCTTCCTGCACCTC
	Reverse	GTACCACTACCACCATTACC
Ccn1	Forward	TACCTACTATTACTCAGTC
	Reverse	GTATAGCTAGAAATAACACC
Clb2	Forward	CCGAATTCATGCCACAAGT
	Reverse	ATGCCACAAGTCACTAAA

4.3. Results and Discussion:

4.3.1. Dysregulation of hyphal and bud-associated transcription factors in *C. albicans* expressing ubiquitin mutations

Real-time PCR analysis was carried out for the detection of the levels of hyphal and bud-associated transcription activators and inhibitors in *C. albicans* expressing ubiquitin mutations. It is evident that both cell cyclins and proteome makeup play key roles in the filamentation of *C. albicans*, and from the previous results in this study it is known that ubiquitin mutants affect morphogenesis and the level of cell cyclins. Secretory aspartyl protease (SAP) is a key enzyme in the establishment of the filamentous form of *C. albicans* in the tissues. It was found from the zymography studies that SAP secretion was affected in *C. albicans* expressing UbEP42, UbL50P and UbI61T.

Further, in the background of ubiquitin mutations, the status of the transcription factors governing morphogenesis is not known. In this study, the mRNA levels of the transcription activators and transcription inhibitors were examined to understand the influence of ubiquitin mutations on the regulation of morphogenesis of *C. albicans* **(Figure 4.1)**. It was reported in the literature that the transcription factor FkH2 is involved in the secretion of SAP. Hence, the mRNA level of FkH2 was monitored and found to be downregulated in UbEP42, UbL50P and UbI61T. Further, certain virulence-associated transcriptional activators and repressors were selected for this study based on the literature, namely Nrg1, Efg1, Rbf1, Rim101 and Tec1 (Sroussi et al., 2017). Nrg1 is an inhibitor of morphogenesis that prevents initiation of hyphal formation. Earlier studies have indicated that to facilitate hyphal growth, Nrg1 is downregulated by cAMP-protein kinase A (PKA) pathway (Lu et al., 2011). Nrg1 is degraded by a mechanism involving kinase Sok1. The transcription repressor Cup9 is known to downregulate the expression of Sok1. However, when conditions promote filamentous growth, Cup9 is broken down after ubiquitination by the ubiquitin ligase (E3) Ubr1, which leads to expression of Sok1. In turn, Sok1 leads to degradation of Nrg1. Removal of Nrg1 subsequently gives rise to hyphal growth (Hossain et al., 2021). The findings in the presence of ubiquitin mutations show a significant increase in Nrg1 transcript levels in UbEP42, UbL50P and UbI61T, suggesting that upregulation of Nrg1 keeps the cells predominantly in their yeast form, negatively impacting their ability to transition into hyphal structures in serum environment.

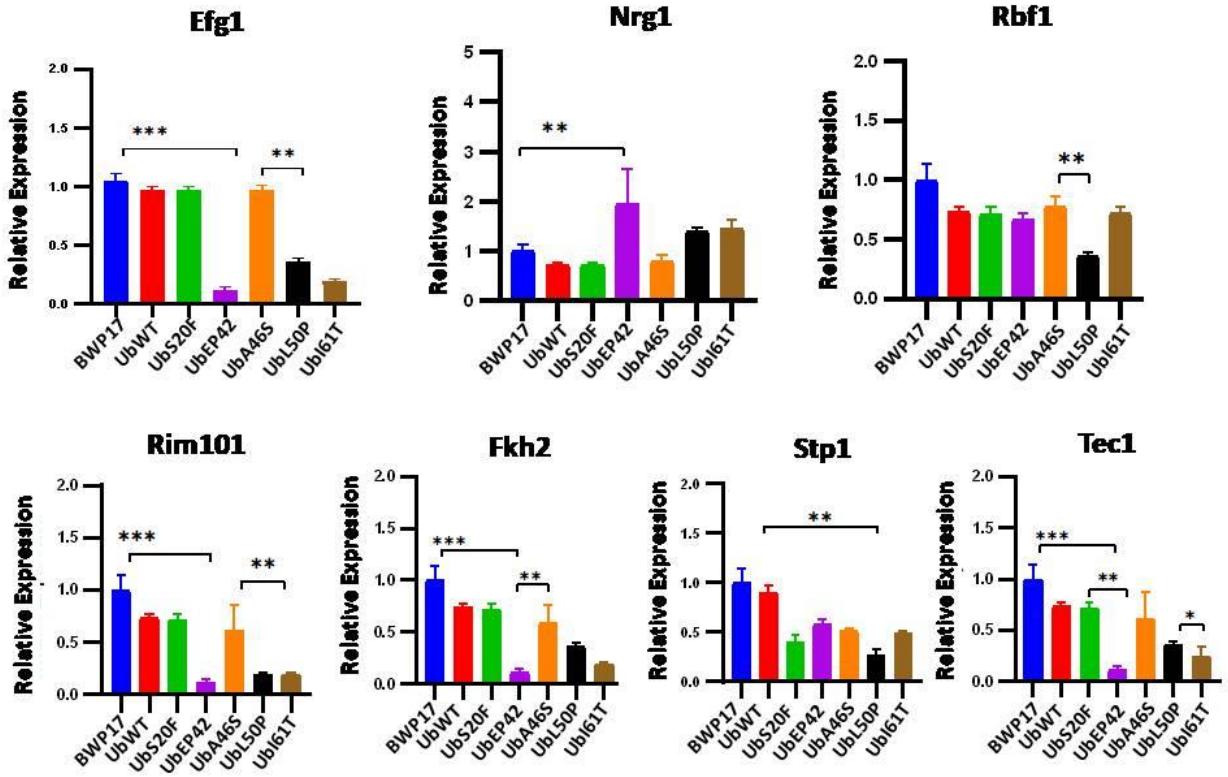


Figure 4.1: Transcription factor m-RNA levels in *C. albicans* under ubiquitin mutants expression. N=3 All values are presented as Mean+ SEM *P<0.05; **P<0.01 and*P<0.001 as compared to control.**

Earlier studies reported that *C. albicans* undergo filamentous growth when exposed to various environmental and host-specific triggers. These signals are processed through intricate and often overlapping signaling pathways, notably the cAMP protein kinase A pathway, which plays a pivotal role in filamentation in response to serum (Hossain et al., 2021), Within this pathway, protein kinase A (PKA) is believed to modify the transcription factor Efg1, triggering a series of genetic responses that control morphogenesis. While Efg1 is crucial for the filamentation process, which is dependent on the cAMP-PKA pathway, it is not necessary for all morphological responses. Here, upon expression of ubiquitin mutations UbEP42, UbL50P and UbI61T, the transcript levels of Efg1 diminished. This suggests potential disruption of the central

cAMP PKA signaling pathway by these mutations. To understand the effect of ubiquitin mutations the levels of four more hyphal-associated transcription factors Rim101, Tec1, Stp1, and Rbf1 were examined in the presence of serum. Rim101, an alkaline-responsive transcription regulator plays an important role in cell wall assembly. Tec1 and Rbf1 regulate hyphal development. Stp1 plays a crucial role in nutrient acquisition. Reduced levels of Rim101, Tec1, and Rbf1 correlated with the expression of the mutations UbEP42, UbL50P, and UbI61T. However, the levels of Stp1, which activates transcription of genes required for extracellular proteolysis and absorption of resultant peptides did not seem to follow any particular trend, unlike the other transcription factors in response to ubiquitin mutations, showing Stp1 levels in UbI61T comparable to that of wild type.

4.3.2. Characterization of Differentially Expressed Proteins in Ubiquitin Mutant Conditions

Raw data from proteomics was analyzed to elucidate the log2 fold change in the expression of differentially expressed proteins in three distinct ubiquitin mutants: UbEP42, UbL50P, and UbI61T. Under the ubiquitin mutant condition, UbEP42 exhibited upregulation of 493 proteins, among which 343 were characterized while 145 remained uncharacterized. Additionally, 128 proteins were found to be downregulated, with 72 characterized and 56 uncharacterized. In the case of UbL50P, 122 proteins were upregulated, with 35 characterized and 87 uncharacterized, while 497 proteins were downregulated, with 347 characterized and 150 uncharacterized. Furthermore, in UbI61T, 160 proteins were upregulated, comprising 53 characterized and 107 uncharacterized proteins. Conversely, 81 proteins were downregulated, with 59 characterized and 22 uncharacterized **(Figure 4.2).** These findings provide insights into the differential expression patterns of proteins under various ubiquitin mutant conditions, shedding light on potential roles and interactions in cellular processes.

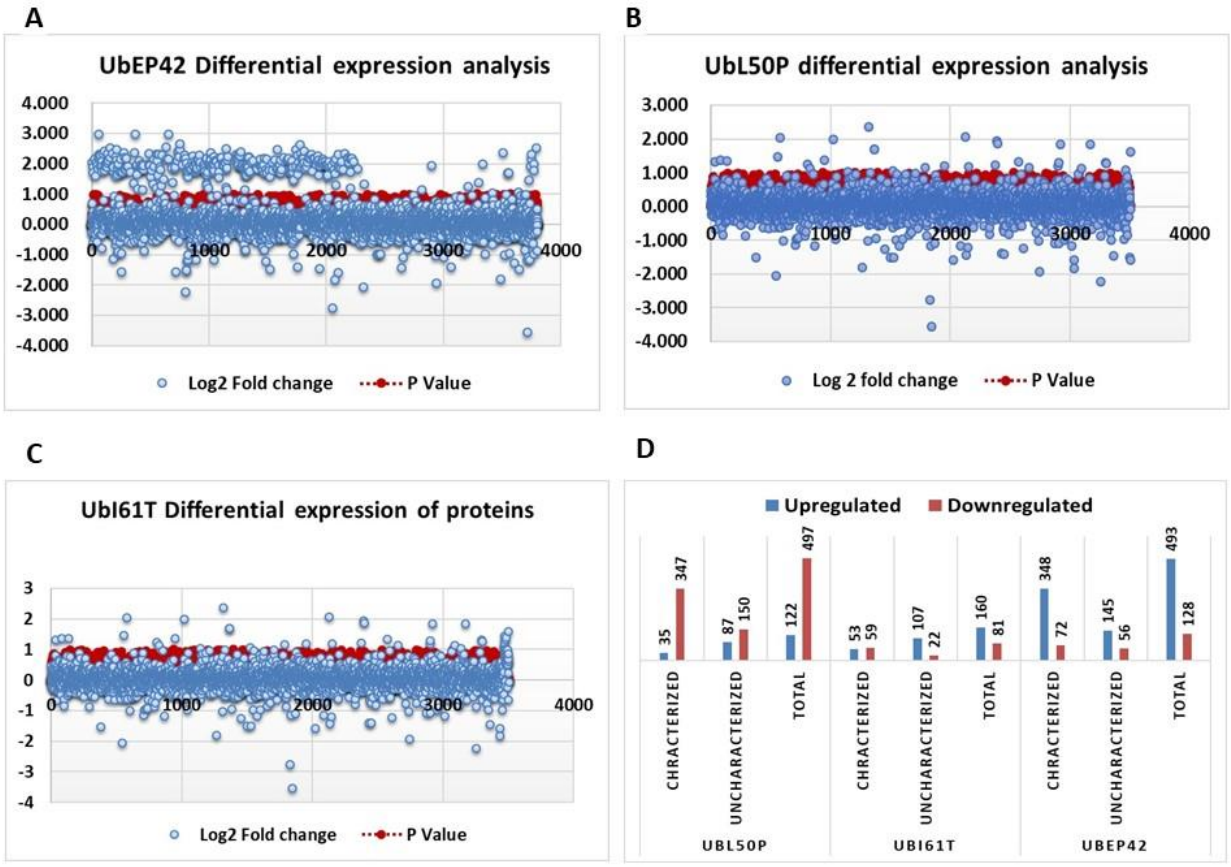


Figure 4.2. Results from DIA quantitation of ubiquitin mutants. Plots (a), (b) and (c) represent the protein profile of upregulated vs downregulated in UbEP42, UbL50P, and UbI61T respectively. Proteins showing an increase which is greater than 1.3-fold (log2 fold change) and a decrease less than 1.3-fold (log2 fold change) were considered and were represented by circular blue dots. Proteins showing (−log10 P value values were represented by red dots (−log10 P value, increase 0.05) or decrease (−log10 P value less than −0.05). Protein abundances in increasing order on the X-axis. Plot (d) represents no. of total proteins that were up and down-regulated in characterized and uncharacterized forms

4.3.3 Common downregulation of some proteins in *Candida albicans* due to Overexpression of Ubiquitin mutants UbL50P, UbI61T, and UbEP42

In the present study, after characterization of total protein up and downregulation, we identified 61 common downregulated individual proteins mentioned in **(Table 4.1)**, that were found altered due to the impact of overexpression of ubiquitin mutants UbEP42, UbL50P, and UbI61T on the proteomic profile of *Candida albicans*.

Table 4.1. Common upregulated and downregulated protein under UbEP42, UbL50P, UbI61T mutants overexpression.

Downregulated in UbEP42,UbL50P,UbI61T					
Uniprot Acce	Column4	Log2F	Column1	Column12	Log2FC C
Q5AI24	3-hydroxyisobutyryl-CoA hydrolase, mitochondrial	-0.547	B9WD01	Myo-inositol transporter, putative	-0.655
C4YIA3	4-aminobutyrate aminotransferase	-0.809	Q59VX9	NAD(P)H-hydrate epimerase	-0.853
A0A454IXA2	Acyl-CoA desaturase	-1.143	Q59T35	Osm1p	-0.582
Q5A1V3	ADP-ribose 1"-phosphate phosphatase	-0.662	A0A454JCN0	Peptidyl-prolyl cis-trans isomerase D	-0.530
A0A454IXD0	Alcohol dehydrogenase 2	-0.968	B9WAG4	Peroxisome assembly protein 12, putative	-0.761
O74626	Alk8 protein	-0.893	A0A1D8PF54	Phosphate transporter	-0.851
A0A454J9J1	Alpha-1,4 glucan phosphorylase	-0.580	C4YNC2	Phosphoenolpyruvate carboxykinase	-1.228
C4YDC2	Alternative oxidase	-1.581	A0A454JBX4	Phosphotransferase (Fragment)	-0.630
Q59N40	Aspartate aminotransferase	-0.552	B9WMS7	Pyridoxal reductase, putative	-0.619
C4YSU5	ATP-dependent (S)-NAD(P)H-hydrate dehydratase	-0.621	Q5AIA6	Pyridoxine biosynthesis protein	-0.525
A0A454J2E0	Beta-glucosidase	-0.559	A0A454JCB2	Pyrimidine biosynthesis enzyme THI13	-0.983
Q59QR8	Bub3p	-0.518	C4YKB8	Respiratory growth induced protein 1	-1.399
Q59Z17	Catabolic 3-dehydroquinase	-0.622	A0A454JCD7	S-formylglutathione hydrolase	-0.547
Q59YD1	Cu/Pi carrier	-0.529	Q5AHH4	Small heat shock protein 21	-1.463
Q5AEN1	Cytochrome c peroxidase, mitochondrial	-0.536	C4YDJ6	Sorting nexin GRD19	-0.614
B9WK31	Eukaryotic translation initiation factor 3 subunit B	-0.547	A0A454IV96	Succinate-semialdehyde dehydrogenase	-0.628
Q5A7S7	Fork-head transcriptional regulator 2	-2.234	B9W6U5	Superoxide dismutase	-0.507
A0A1D8PNS1	Formate dehydrogenase	-1.521	Q5APC5	SURF1-like protein	-0.569
C4YF57	GAL10 bifunctional protein	-1.216	Q5A7P9	Thioredoxin peroxidase	-0.870
P56091	Galactokinase	-1.172	B9WH78	Thioredoxin, putative	-2.770
A0A454IVM3	Galactose-1-phosphate uridylyltransferase	-1.008	Q5ANJ4	Transcription activator TEC1	-1.826
A0A454IVV2	Glucan 1,3-beta-glucosidase	-0.577	Q59LU6	Triglyceride lipase	-0.647
Q5BM34	Glucosamine-6-phosphate isomerase (Fragment)	-0.643	C4YEH1	Urea amidolyase	-1.165
A0A454IYA9	Glutamate decarboxylase	-0.696	B9W7C4	Vanillin dehydrogenase, putative	-0.973
Q5AKX1	Glycine cleavage system H protein	-0.607	Upregulated in UbEP42,UbL50P,UbI61T		
A5GXM4	Glycoamidase	-1.177	A0A454IV45	1,3-beta-glucanosyltransferase	2.034
A0A454JA40	Glycogen [starch] synthase	-0.556	Q59PF0	Elongation of fatty acids protein	0.943
Q5AFB4	Gst2p	-0.765	C5K446	High-affinity glucose transporter	1.293
C4YFZ1	Heat shock protein SSA4	-0.635	Q8TG35	Mnn4p	0.834
Q5AD47	Hexose transporter	-0.657	C4YM00	Nucleolar complex protein 2	0.81
C4YCX1	Hit family protein 1	-0.535	C4YJR4	Ribonucleoside-diphosphate reductase	1.867
Q59NN8	Hsp70 nucleotide exchange factor FES1	-0.584	Q9Y872	Sulfate adenylyltransferase	1.614
A0A454IZV7	Hsp78-like protein	-1.174	A0A1D8PIF6	Sulfite reductase subunit alpha	0.884
A0A454J835	Hsp90-like protein	-0.547	Q5AB97	Hmx1p	0.914
C4YEI7	Isocitrate lyase	-0.747	A0A454J674	Inositol phosphorylceramide synthase	0.956
Q5APD2	Malate synthase	-0.501	Q00312	Transcription factor Rbf1	1.303
Q9UW14	pH-response transcription factor pacC/RIM101)	-1.500	Q5A0E5	Transcriptional regulator NRG1 1	1.848

Table 4.2. Upregulated and Downregulated protein associated with biological process and metabolic pathway under UbEP42, UbL50P, UbI61T mutants expression.

Downregulated protein associated with biological process under Ub mutants UbEP42, UbL50P, UbI61T

Biological Process (Gene Ontology)			
description	count in network	strength	false discovery rate
Glucan catabolic process	3 of 8	1.71	0.0155
Energy reserve metabolic process	3 of 8	1.71	0.0155
Cellular aldehyde metabolic process	4 of 15	1.56	0.0033
Cellular detoxification	4 of 33	1.22	0.0284
Cellular glucan metabolic process	4 of 34	1.21	0.0299
Cellular amino acid catabolic process	5 of 48	1.15	0.0104
Carbohydrate catabolic process	5 of 57	1.08	0.0170
Small molecule catabolic process	10 of 128	1.03	0.00013
Carboxylic acid catabolic process	7 of 89	1.03	0.0026
Cellular carbohydrate metabolic process	8 of 111	0.99	0.0014
Carbohydrate metabolic process	10 of 186	0.87	0.0012
Monocarboxylic acid metabolic process	7 of 137	0.85	0.0161
Oxoacid metabolic process	13 of 363	0.69	0.0014
Carboxylic acid metabolic process	12 of 353	0.67	0.0029
Small molecule metabolic process	18 of 614	0.6	0.00023
Organic substance catabolic process	14 of 538	0.55	0.0068
Catabolic process	15 of 617	0.52	0.0068
Cellular catabolic process	12 of 516	0.5	0.0452

Downregulated protein associated with metabolism under Ub mutants UbEP42, UbL50P, UbI61T

KEGG Pathways			
description	count in network	strength	false discovery rate
Butanoate metabolism	3 of 14	1.47	0.0038
Tyrosine metabolism	3 of 19	1.34	0.0073
Glyoxylate and dicarboxylate metabolism	4 of 26	1.32	0.0017
Alanine, aspartate and glutamate metabolism	4 of 27	1.31	0.0017
beta-Alanine metabolism	3 of 20	1.31	0.0074
Starch and sucrose metabolism	4 of 32	1.23	0.0023
Carbon metabolism	8 of 98	1.05	4.07e-05
Biosynthesis of secondary metabolites	11 of 322	0.67	0.00067
Metabolic pathways	22 of 785	0.58	5.37e-07

Upregulated protein associated with metabolism under Ub mutants UbEP42, UbL50P, UbI61T

KEGG Pathways				
pathway	description	count in network	strength	false discovery rate
cal00920	Sulfur metabolism	2 of 14	1.94	0.0345

Through proteomic analysis, we identified a multitude of proteins that exhibited downregulation upon overexpression of these ubiquitin mutants. These proteins play crucial roles in various cellular biological processes, including metabolism, stress response, protein folding, cell wall

synthesis, and secretion of aspartyl protease **Table 4.2.** Downregulated proteins associated with biological processes and metabolic pathways are illustrated in **Table 4.2**. Further, we shed light on their importance and how they are interconnecting in biological and metabolic pathways to understand their virulence under particular protein level

4.3.4. Metabolic Pathways and Stress Response

Several enzymes involved in metabolic pathways were found to be downregulated in response to the overexpression of ubiquitin mutants. The downregulation of these enzymes suggests a potential disruption in key metabolic processes such as fatty acid metabolism, amino acid metabolism and carbohydrate metabolism. Moreover, the downregulation of stress response proteins such as heat shock proteins (Hsp70, Hsp78-like, Hsp90-like) (Gong et al., 2017), fork-head transcriptional regulator 2 (Bensen et al., 2002), and respiratory growth-induced protein 1 (Abdhulghani et al., 2022). This list of downregulated proteins indicates the compromised status of the cell to cope with environmental stress. This downregulation may contribute to the observed decrease in thermotolerance and antibiotic stress resistance in *C. albicans* overexpressing ubiquitin mutants (Dantuslia et al., 2023.

4.3.5. Cell Wall Synthesis and Morphogenesis

C. albicans cell wall integrity is crucial for its survival and pathogenesis. Proteomics results revealed the downregulation of proteins involved in cell wall synthesis such as GPI-anchored proteins, β-1,3-glucan synthase could lead to reduced synthesis of β-glucan, resulting in altered cell wall composition and increased exposure of existing β-glucans (De Assi et al., 2022). However, other down regulated proteins such as α-1,4 glucan phosphorylase, glucan 1,3-β-glucosidase, Glucosamine-6-phosphate isomerase (fragment), hexose transporter, pH-response transcription factor pacC/RIM101, myo-inositol transporter, putative peroxisome assembly protein 12 and putative Phosphate transporter sorting nexin GRD19 are involved in various aspects of cell wall synthesis, organization, and maintenance in C. albicans. It is important to note that some proteins may have indirect roles in cell wall maintenance or may participate in multiple cellular processes. Changes in their levels also could compromise the structural integrity of the cell wall, rendering *C. albicans* more susceptible to host immune responses and antifungal treatments.

4.3.6. Protein Folding and Ubiquitin-Mediated Proteolysis

The observed downregulation of molecular chaperones in the presence of ubiquitin mutations itself suggests impairment in protein folding and failure of quality control mechanisms go hand in hand. Additionally, the downregulation of ADP-ribose 1'-phosphate phosphatase may disrupt ubiquitin-mediated proteolysis and protein degradation pathways in yeast (Verheugd et al., 2016). This dysregulation could lead to the accumulation of misfolded or damaged proteins, further exacerbating cellular stress and compromising viability.

4.3.7. Secretion of Aspartyl Protease

Interestingly, proteomics analysis of cells overexpressing ubiquitin mutants revealed a decrease in the levels of forkhead transcription factor 2. The transcription factor can either activate or repress the expression of target genes, thereby influencing cellular processes, including protein secretion. Therefore, Forkhead transcription factor 2 (Fork-head transcriptional regulator 2) may indirectly regulate the secretion of aspartyl proteases in *C. albicans* directly regulating the expression of genes encoding secretory aspartyl protease or by controlling the expression of genes involved in the secretory pathway in *C. albicans* (Greig et al., 2015). In our study it was observed that overexpressing ubiquitin mutants leads to a decreased level of aspartyl protease secretion (Sandeep Kumar Datuslia, Bhoomi Prajapati and C. Ratna Prabha, manuscript under communication). However, it is an interesting novel observation that ubiquitination has importance in protease secretion. Further investigations are required to explain how ubiquitination influences FKh2 transcription factor bringing about the regulation of aspartyl protease secretion.

4.3.8. Pathways Affected by Downregulated Proteins

The downregulation of various proteins identified in our study explains potential dysregulation in multiple cellular pathways in *C. albicans*. Specifically, the affected proteins are involved in crucial processes such as carbohydrate metabolism, stress response, cell wall synthesis and the expression of virulence factors. Proteins implicated in carbohydrate metabolism, including pyruvate carboxykinase and hexose transporter are downregulated, indicating potential alterations in glucose utilization and energy production pathways. Heat shock proteins (HSPs)

such as Hsp70 and Hsp90-like proteins, crucial for stress response and protein folding, exhibit reduced expression levels in ubiquitin mutants of *C. albicans*, suggesting impairment in the ability to cope with environmental stresses. Downregulation of enzymes involved in cell wall synthesis pathways, such as α-1,4 glucan phosphorylase and glucosamine-6-phosphate isomerase, implies potential disruptions in cell wall integrity, leading to increased exposure of β-glucan and enhanced chitin deposition. Decreased expression levels of secretory aspartyl proteases (SAPs), which are important virulence factors, indicate a potential attenuation of virulence in *C. albicans* upon overexpression of ubiquitin mutants. The downregulation of these proteins collectively impacts several vital pathways in *C. albicans*. These include but are not limited to, carbohydrate metabolism, lipid metabolism, stress response, cell wall synthesis, protein quality control and virulence factor secretion. Dysregulation of these pathways could contribute to the observed phenotypic changes, including decreased cell viability, impaired thermotolerance, increased susceptibility to antibiotic stress, and attenuated virulence.

4.3.9. Up and downregulated protein-protein functional interactions as revealed by String node interaction

To identify the interaction of a protein in a biological process and/ or a metabolism, differentially expressed downregulated proteins common among three ubiquitin mutants UbEP42, UbL50P, and UbI61T in a consensus module were input into the STRING database, where interactions with a high confidence score of ≥ 0.7 were considered to minimize false-positive results. Subsequently, the network-generated output is presented in **(Figure 4.3),** for the biological processes mentioned in **Table 4.2,** and the involvement of downregulated proteins in the metabolic pathway is given in **(Figure 4.4).**

Glycolysis and gluconeogenesis pathways were selected to study further as the scores of the proteins belonging to these pathways were on the higher side. KEGG Glycolysis and gluconeogenesis pathways database analysis was done in Cytoscape to check the involvement of the downregulated proteins in the pathway **(Figure 4.5)**. Similar exercise was repeated with 12 common upregulated proteins in *C. albicans* cells expressing ubiquitin mutants UbEP42, UbL50P, and and UbI61T and it was found that only two proteins MET3, MET10 showed close functional interaction **(Figure 4.6)**.

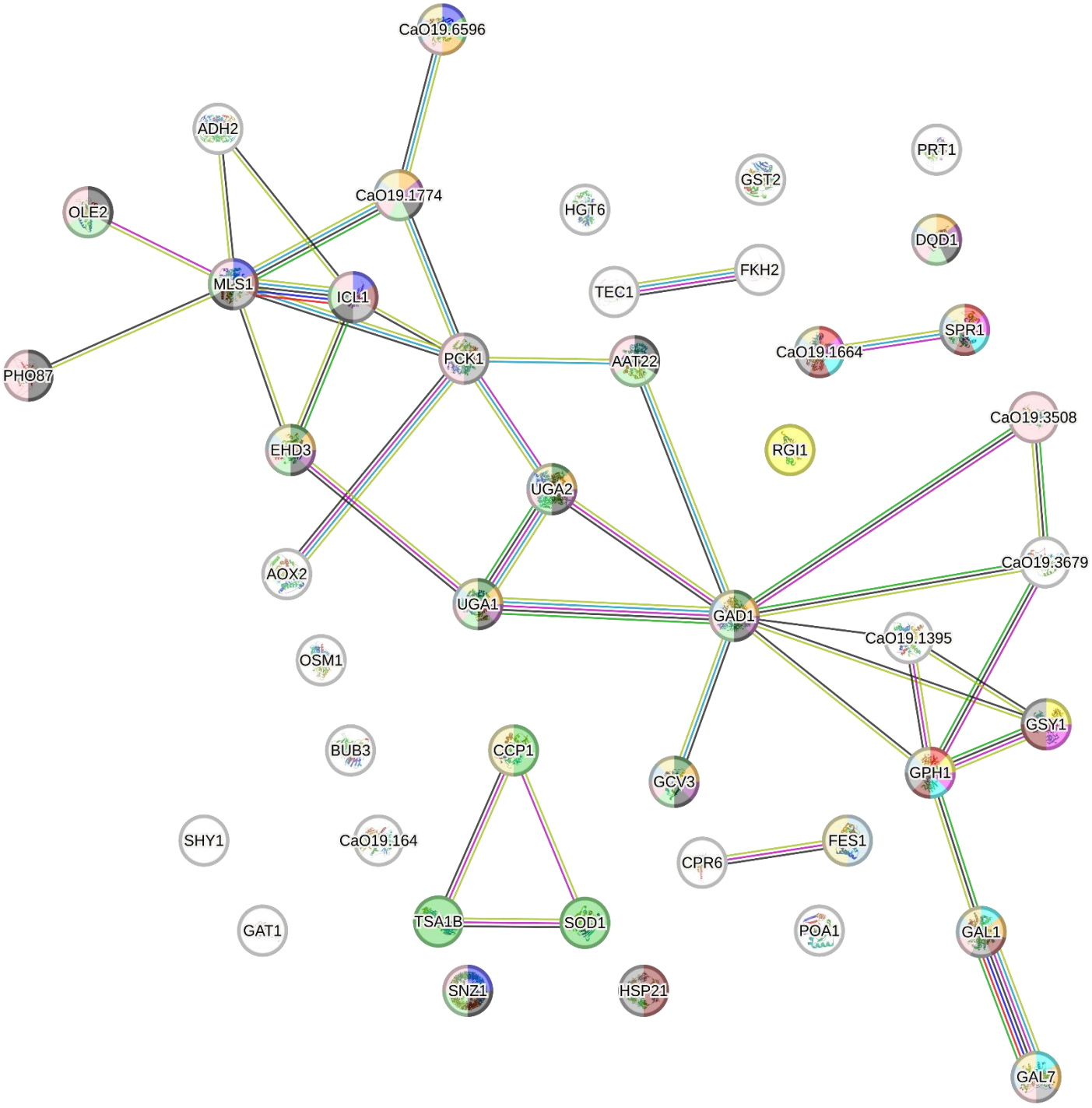


Figure 4.3. STRING analysis of differentially regulated proteins involved in the biological processes of C. albicans due to the expression of ubiquitin mutants. Protein-protein functional interaction prediction map of downregulated proteins of *C. albicans* obtained using STRING v.11 database (confidence score 0.700).

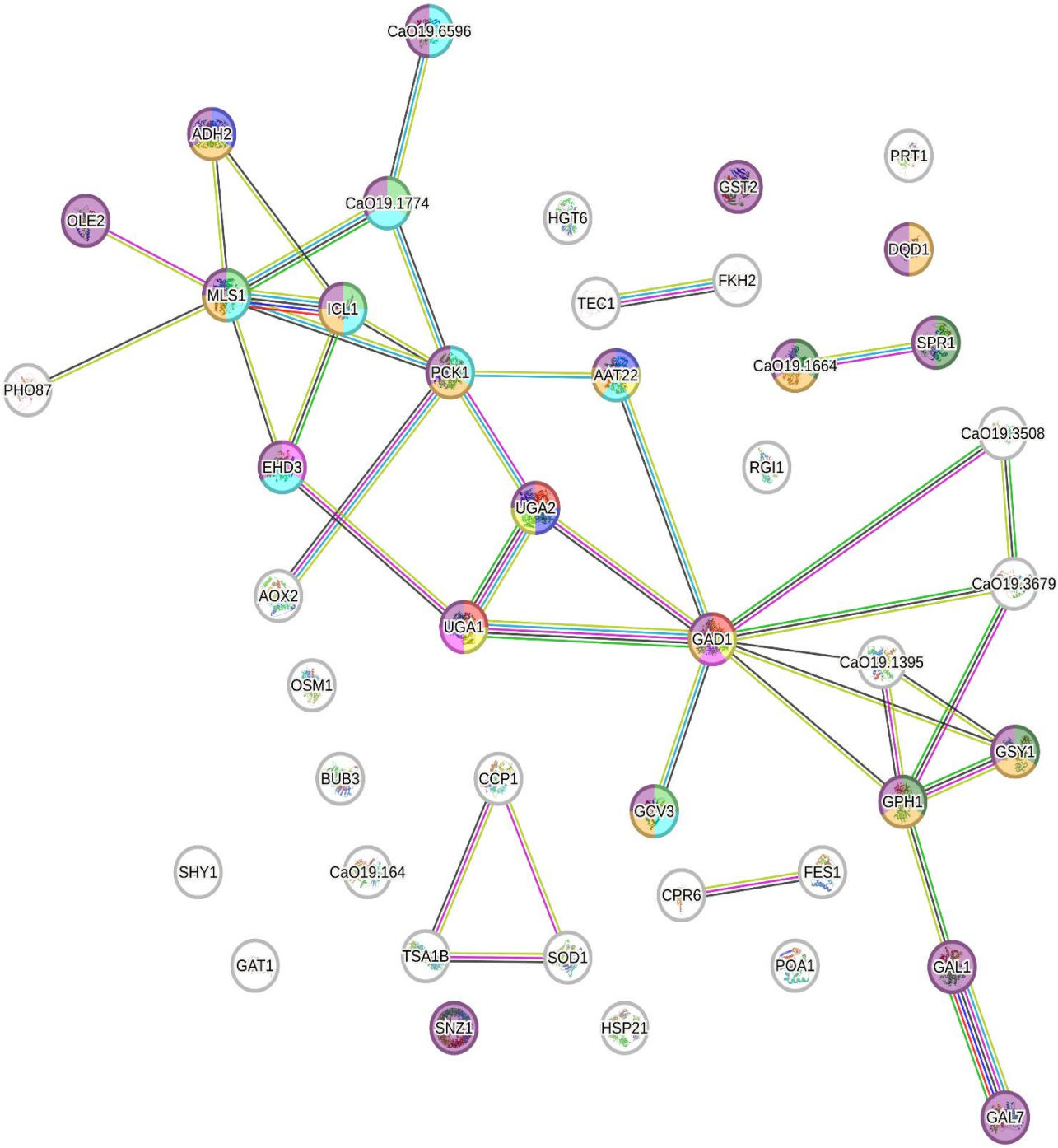
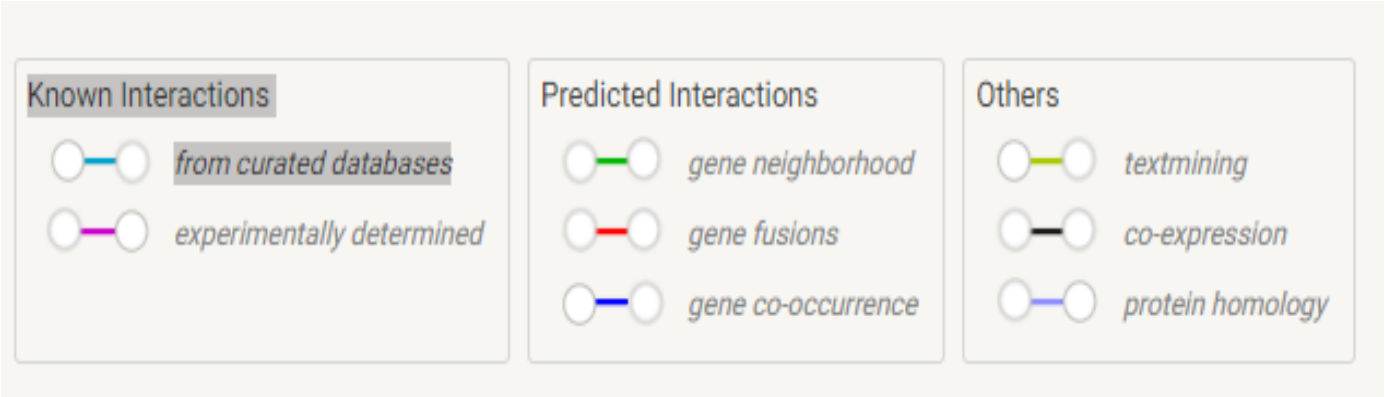


Figure 4.4. STRING analysis of differentially regulated proteins involved in the metabolic processes of C. albicans due to the expression of ubiquitin mutants. Protein-protein functional interaction prediction map of downregulated proteins of C. albicans obtained

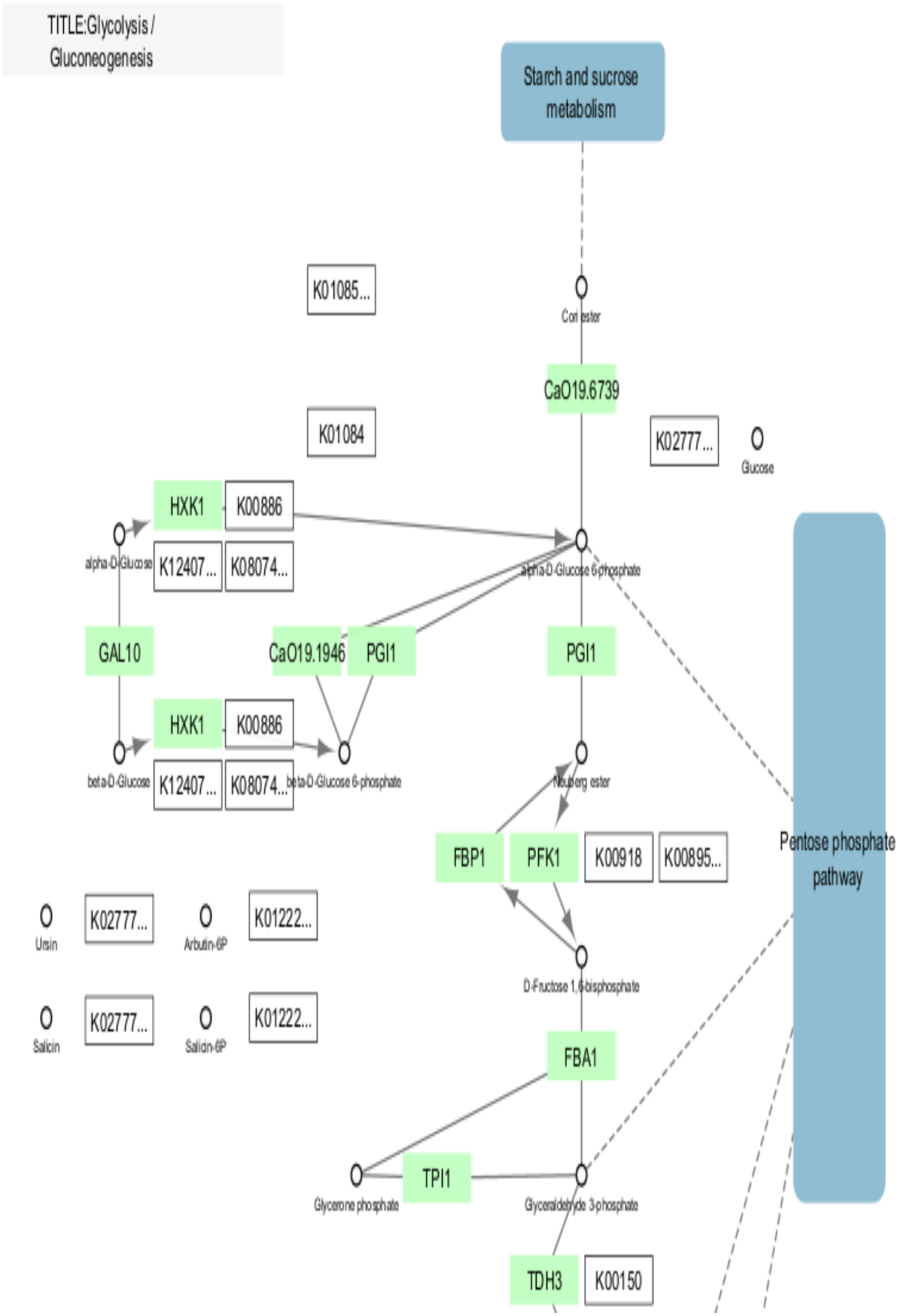
using STRING v.11 database (confidence score 0.700). Filled protein nodes that signify the availability of protein 3D structural information either known or predicted.

{Annotation of Gene Interaction}



HMX1	*Hmx1p. (291 aa)*
MET3	*Sulfate adenylyltransferase; Catalyzes the first intracellular reaction of sulfate assimilation, forming adenosine-5'-phosphosulfate (APS) from inorganic sulfate and ATP. Plays an important role in sulfate activation as a component of the biosynthesis pathway of sulfur-containing amino acids. Belongs to the sulfate adenylyltransferase family. (527 aa)*
MET10	*Sulfite reductase subunit alpha. (1094 aa)*
MNN4	*Mnn4p. (996 aa)*
PHR3	*1,3-beta-glucanosyltransferase; Splits internally a 1,3-beta-glucan molecule and transfers the newly generated reducing end (the donor) to the non-reducing end of another 1,3-beta-glucan molecule (the acceptor) forming a 1,3-beta linkage, resulting in the elongation of 1,3-beta-glucan chains in the cell wall; Belongs to the glycosyl hydrolase 72 family. (487 aa)*
HGT1	*High-affinity glucose transporter 1; High-affinity glucose transporter. Acts as a multifunctional complement-evasion molecule that causes down-regulation of complement activation by acquisition of human complement factors FH and C4BP. Functions also as a human immunodeficiency virus (HIV) receptor via binding the viral gp160 protein. Modulates hyphae formation. (545 aa)*
FEN12	*Elongation of fatty acids protein. (292 aa)*
RNR3	*Ribonucleoside-diphosphate reductase; Provides the precursors necessary for DNA synthesis. Catalyzes the biosynthesis of deoxyribonucleotides from the corresponding ribonucleotides. (816 aa)*
RBF1	*Transcription factor RBF1; Transcriptional activator that binds to the RPG box and to telomeres. Involved in the regulation of the transition between yeast and filamentous forms and plays a role in virulence. Induces expression of HWP1, a major hyphal cell protein and virulence factor. Belongs to the RBF1 family. (534 aa)*
NOC2	*mRNA-binding ribosome synthesis protein. (714 aa)*

AOX2	*Alternative oxidase. (365 aa)*
RGI1	*Respiratory growth induced protein 1; Involved in the control of energetic metabolism and significantly contribute to cell fitness, especially under respiratory growth conditions. (201 aa)*
ICL1	*Isocitrate lyase. (550 aa)*
AAT22	*Aspartate aminotransferase. (409 aa)*
GAD1	*Glutamate decarboxylase; Belongs to the group II decarboxylase family. (568 aa)*
EHD3	*3-hydroxyisobutyryl-CoA hydrolase, mitochondrial; Hydrolyzes 3-hydroxyisobutyryl-CoA (HIBYL-CoA), a saline catabolite; Belongs to the enoyl-CoA hydratase/isomerase family. (502 aa)*
CaO19.1774	*Formate dehydrogenase; Catalyzes the NAD(+)-dependent oxidation of formate to carbon dioxide. Formate oxidation is the final step in the methanol oxidation pathway in methylotrophic microorganisms. Has a role in the detoxification of exogenous formate in non-methylotrophic organisms. Belongs to the D-isomer specific 2-hydroxyacid dehydrogenase family. FDH subfamily. (380 aa)*
BUB3	*Bub3p. (373 aa)*
GPH1	*Alpha-1,4 glucan phosphorylase; Phosphorylase is an important allosteric enzyme in carbohydrate metabolism. Enzymes from different sources differ in their regulatory mechanisms and in their natural substrates. However, all known phosphorylases share catalytic and structural properties. (900 aa)*
SPR1	*Spr1p. (525 aa)*
GAL7	*Galactose-1-phosphate uridylyltransferase; Belongs to the galactose-1-phosphate uridylyltransferase type 1 family. (386 aa)*
SHY1	*SURF1-like protein; Probably involved in the biogenesis of the COX complex. Belongs to the SURF1 family. (359 aa)*
UGA2	*Succinate-semialdehyde dehydrogenase. (509 aa)*
GST2	*Gst2p; Belongs to the GST superfamily. (219 aa)*
FKH2	*Fork-head transcriptional regulator 2; Transcription factor required for the morphogenesis of true hyphal as well as yeast cells. Contributes to virulence. (687 aa)*
TSA1B	*Peroxiredoxin TSA1-B; Thiol-specific peroxidase that catalyzes the reduction of hydrogen peroxide and organic hydroperoxides to water and alcohols, respectively. Plays a role in cell protection against oxidative stress by detoxifying peroxides and as sensor of hydrogen peroxide-mediated signaling events. Also involved in the correct composition of the hyphal cell wall. (196 aa)*
CCP1	*Cytochrome c peroxidase, mitochondrial; Destroys radicals which are normally produced within the cells and which are toxic to biological systems. (366 aa)*
CaO19.3679	*NAD(P)H-hydrate epimerase; Catalyzes the epimerization of the S- and R-forms of NAD(P)HX, a damaged form of NAD(P)H that is a result of enzymatic or heat-dependent hydration. This is a prerequisite for the S-specific NAD(P)H-hydrate dehydratase to allow the repair of both epimers of NAD(P)HX. (258 aa)*
GAT1	*Transcriptional regulatory protein GAT1; Transcriptional regulator of nitrogen utilization required for nitrogen catabolite repression and utilization of isoleucine, tyrosine and tryptophan as nitrogen sources. Controls expression of the MEP2 ammonium permease, the DUR1,2 urea amidolyase, and the transcription factor STP1, which in turn mediates SAP2 expression, a long-known virulence attribute of C.albicans. Influences the filamentation process depending upon the nitrogen sources available. Required for virulence in a mouse systemic infection model. (688 aa)*
HSP21	*Small heat shock protein 21; Heat shock protein required for pathogenicity. Mediates thermotolerance and adaptation to oxidative stress and ethanol-induced stress. Required for invasive growth and filament formation under various filament inducing conditions. Plays a role in the capacity of damaging human-derived endothelial and oral epithelial cells during infection. Potentiates resistance to antifungal drugs, as well as resistance to killing by human neutrophils. Plays a major role in trehalose homeostasis in response to elevated temperatures. Regulates CEK1 activation by phosphoryla [...] (189 aa)*
PRT1	*Eukaryotic translation initiation factor 3 subunit B; RNA-binding component of the eukaryotic translation initiation factor 3 (eIF-3) complex, which is involved in protein synthesis of a specialized repertoire of mRNAs and, together with other initiation factors, stimulates binding of mRNA and methionyl-tRNAi to the 40S ribosome. The eIF-3 complex specifically targets and initiates translation of a subset of mRNAs involved in cell proliferation. (739 aa)*
MLS1	*Malate synthase; Belongs to the malate synthase family. (551 aa)*
CaO19.1395	*Cu/Pi carrier; Belongs to the mitochondrial carrier (TC 2.A.29) family. (338 aa)*
CPR6	*Peptidyl-prolyl cis-trans isomerase D; PPIases accelerate the folding of proteins. It catalyzes the cis-trans isomerization of proline imidic peptide bonds in oligopeptides (By similarity). (369 aa)*
GAL1	*Galactokinase. (515 aa)*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Starch and sucrose metabolism
K01085...
Corn ester
CaO19.6739
K01084
K02777...
Glucose
HXK1
K00886
alpha-D-Glucose
K12407...
K08074...
alpha-D-Glucose 6-phosphate
GAL10
CaO19.1946
PGI1
PGI1
HXK1
K00886
beta-D-Glucose
K12407...
K08074...
beta-D-Glucose 6-phosphate
Neuberg ester
FBP1
PFK1
K00918
K00895...
Pentose phosphate pathway
Ursin
K02777...
Arbutin-6P
K01222...
D-Fructose 1,6-bisphosphate
Salicin
K02777...
Salicin-6P
K01222...
FBA1
TPI1
Glycerone phosphate
Glyceraldehyde 3-phosphate
TDH3
K00150

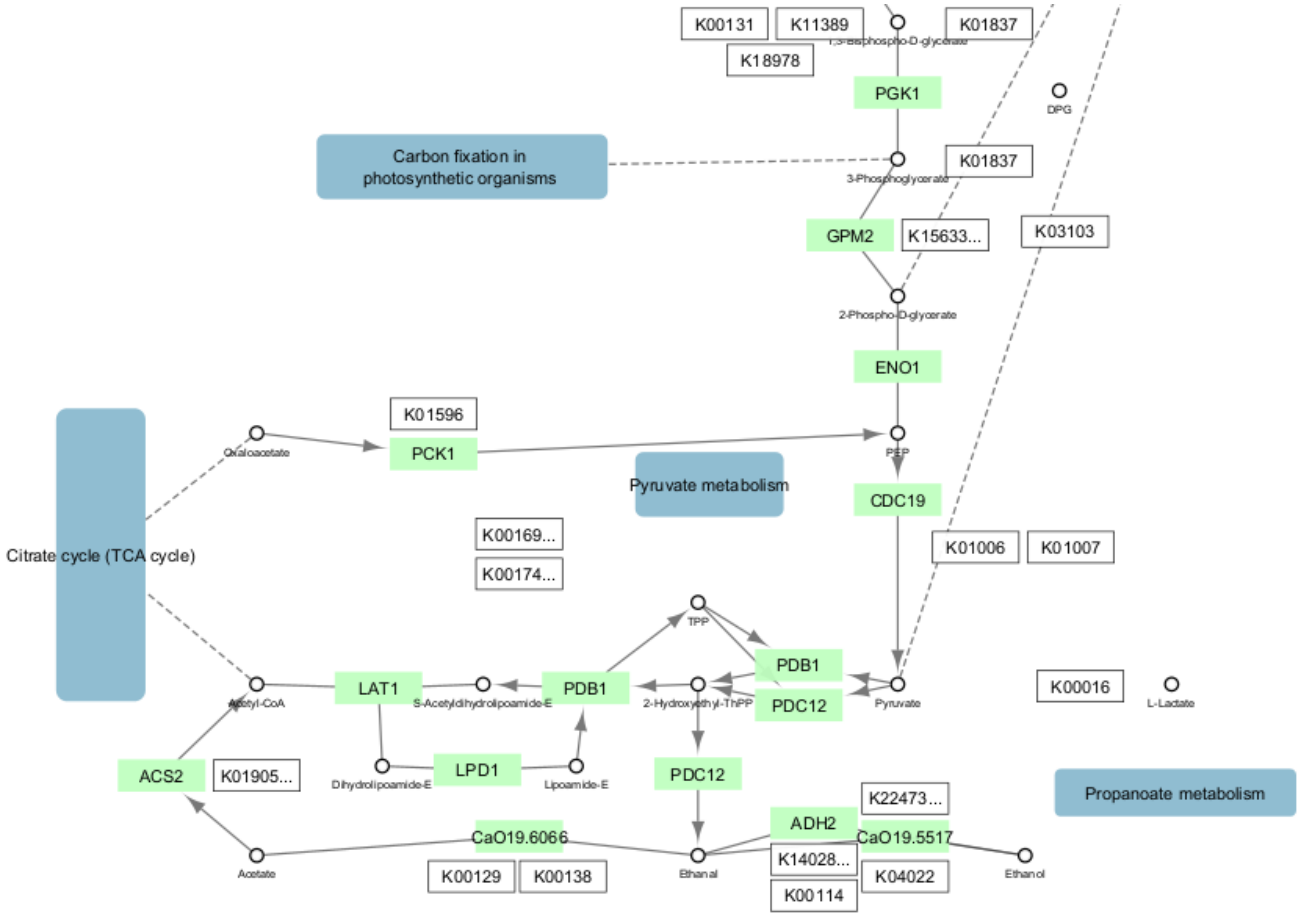


Figure 4.5. KEGG pathway map of glycolysis/ gluconeogenesis. Mappable enzymes within the pathway are highlighted in green and teal denoted as the pathways identified with the aid of the following database: https://www.kegg.jp/entry/cal00010 through http://www.cytoscape

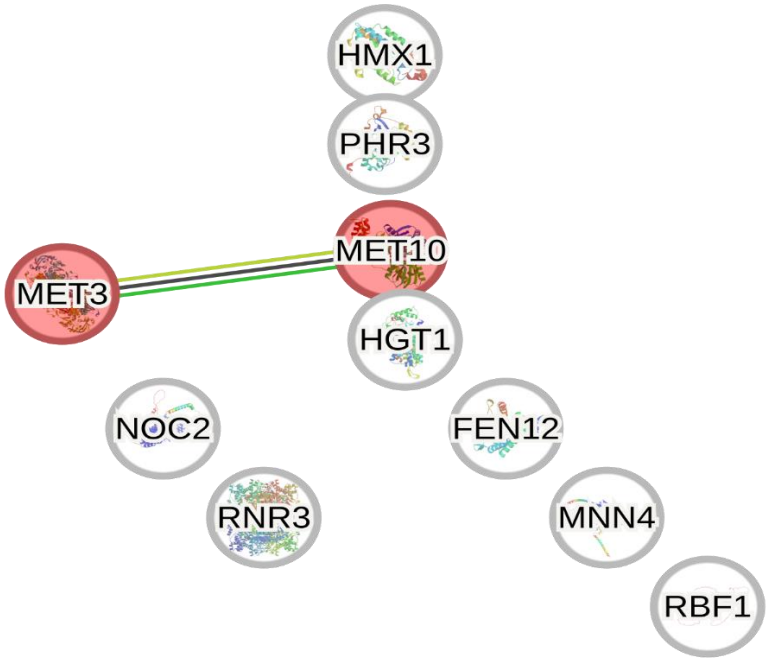
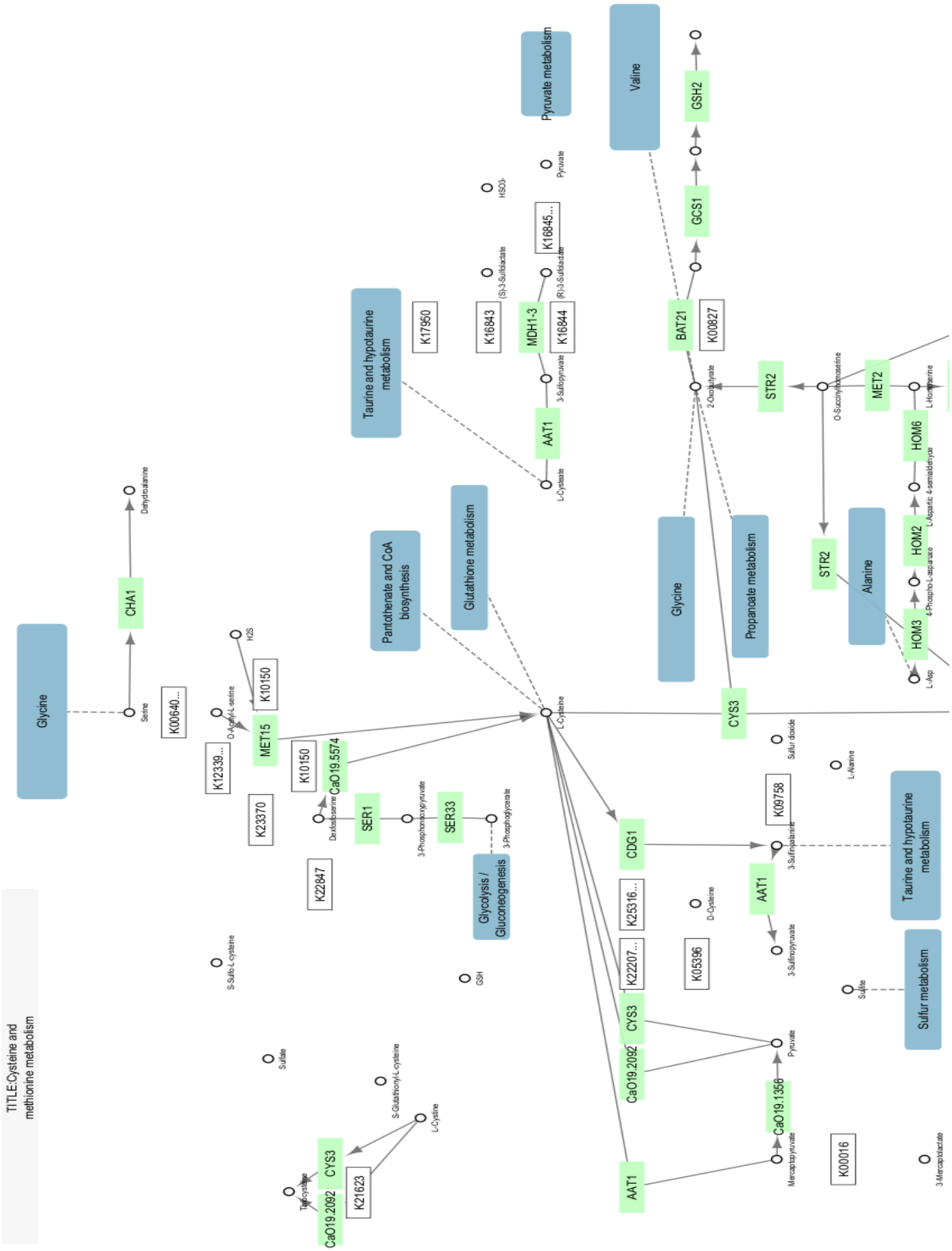


Figure 4.6. Gene ontology and STRING analysis of differentially regulated proteins involved in the metabolic processes of C. albicans due to the expression of ubiquitin mutants. Protein-protein functional interaction prediction map of upregulated proteins of C. albicans obtained using STRING v.11 database (confidence score 0.700). Red color indicates the interaction of MET3 with MET10.

4.3.10. Identification of common upregulated proteins in *Candida albicans* expressing UbEP42, UbL50P, and UbI61T ubiquitin mutants:

Proteomic analysis of *C. albicans* under the influence of UbEP42, UbL50P and UbI61T ubiquitin mutants revealed a significant upregulation of several proteins associated with various cellular processes. There were 12 common proteins which were upregulated among UbEP42, UbL50P, and UbI61T mentioned in **Table 4.1**. These upregulated proteins play crucial roles in modulating the cell cycle, viability, ubiquitin chain formation and stress tolerance in *C. albicans*. Among the upregulated proteins, transcription factors such as Rbf1 and NRG1 were identified, indicating their potential impact on cell cycle regulation and gene expression control (Dhillon et al., 2003). Rbf1 is known to regulate the G1 to S phase transition in the cell cycle, while Nrg1 is

involved in the repression of filamentation and virulence genes. Interestingly Rbf1 and Nrg1 produce opposing effects with the cell cycle. The upregulation of these transcription factors may lead to dysregulation of cell cycle progression and contribute to decreased viability in *C. albicans* under the influence of ubiquitin mutations. The upregulation of proteins involved in ubiquitin signaling, such as 1,3-β-glucanosyltransferase, suggests potential alterations in ubiquitin chain formation and protein degradation processes (Cao et al., 2021)**.** Ubiquitin mutants may contaminate the pool of ubiquitin inside the cell and disrupt the formation of K48 and K63 polyubiquitin chain types, leading to impaired protein turnover and cellular homeostasis. This dysregulation in ubiquitin signaling pathways could further exacerbate cellular stress responses and compromise viability. The upregulation of stress response proteins, including elongation of fatty acids protein FEN12 and high-affinity glucose transporter HGT1 **(Table 4.1)**, indicates a compensatory response to the stress imposed by ubiquitin mutant overexpression (Brown et al., 2006). Through string analysis, it was found that only two MET3 and MET10 have better interactive scores than others **(Figure 4.6)**. MET3 and MET10 represent sulfate adenylyl transferase and sulfate reductase respectively, which are involved in the sulfur metabolism pathway **(Table 4.1**). MET3 Sulfate adenylyl transferase Catalyzes the first intracellular reaction of sulfate assimilation, forming adenosine-5'-phosphosulfate (APS) from inorganic sulfate and ATP. Plays an important role in sulfate activation as a component of the biosynthesis pathway of sulfur-containing amino acids. APS belongs to the sulfate adenylyl transferase family. These proteins enhance antibiotic and heat stress tolerance in *C. albicans*, suggesting a protective mechanism to counteract the deleterious effects of ubiquitin dysregulation on fungal viability and survival observed previously (Dantuslia et al., 2023). Further to identify up and down-regulated proteins in the cysteine methionine pathway the cal00270 KGML file of cysteine methionine pathway was obtained from the KEGG supportive database. The file was uploaded for analysis through String database, for Cytoscape validation. The results obtained are supportive of the functional involvement of MET3 and MET10 in the cysteine methionine pathway **(Figure 4.7)**.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Glycine
CHA1
Dehydroalanine
Serine
K00640...
K12339...
O-Acetyl-L-serine
MET15
H2S
K10150
K10150
K23370
K22847
CaO19.5574
Dehydroalanine
SER1
3-Phosphohydroxypyruvate
SER33
3-Phosphoglycerate
Glycolysis / Gluconeogenesis
Pantothenate and CoA biosynthesis
Glutathione metabolism
Taurine and hypotaurine metabolism
K17950
K16843
(S)-3-Sulfolactate
MDH1-3
K16844
K16845...
(R)-3-Sulfolactate
Pyruvate metabolism
HSO3-
Pyruvate
AAT1
3-Sulfopyruvate
L-Cysteine
Valine
BAT21
K00827
GCS1
GSH2
2-Oxobutyrate
Glycine
Propanoate metabolism
CYS3
L-Cysteine
STR2
O-Succinylhomoserine
MET2
L-Homoserine
STR2
Alanine
HOM6
HOM2
L-Aspartic 4-semialdehyde
HOM3
4-Phospho-L-aspartate
L-Asp
S-Sulfo-L-cysteine
GSH
Sulfate
S-Glutathionyl-L-cysteine
L-Cystine
Thiocysteine
CaO19.2092
CYS3
K21623
CDG1
K25316...
K22207...
CYS3
CaO19.2092
AAT1
K09758
3-Sulfinoalanine
AAT1
3-Sulfinopyruvate
K05396
D-Cysteine
Sulfur dioxide
L-Alanine
Taurine and hypotaurine metabolism
Sulfur metabolism
Sulfite
CaO19.1356
Mercaptopyruvate
Pyruvate
K00016
3-Mercaptolactate

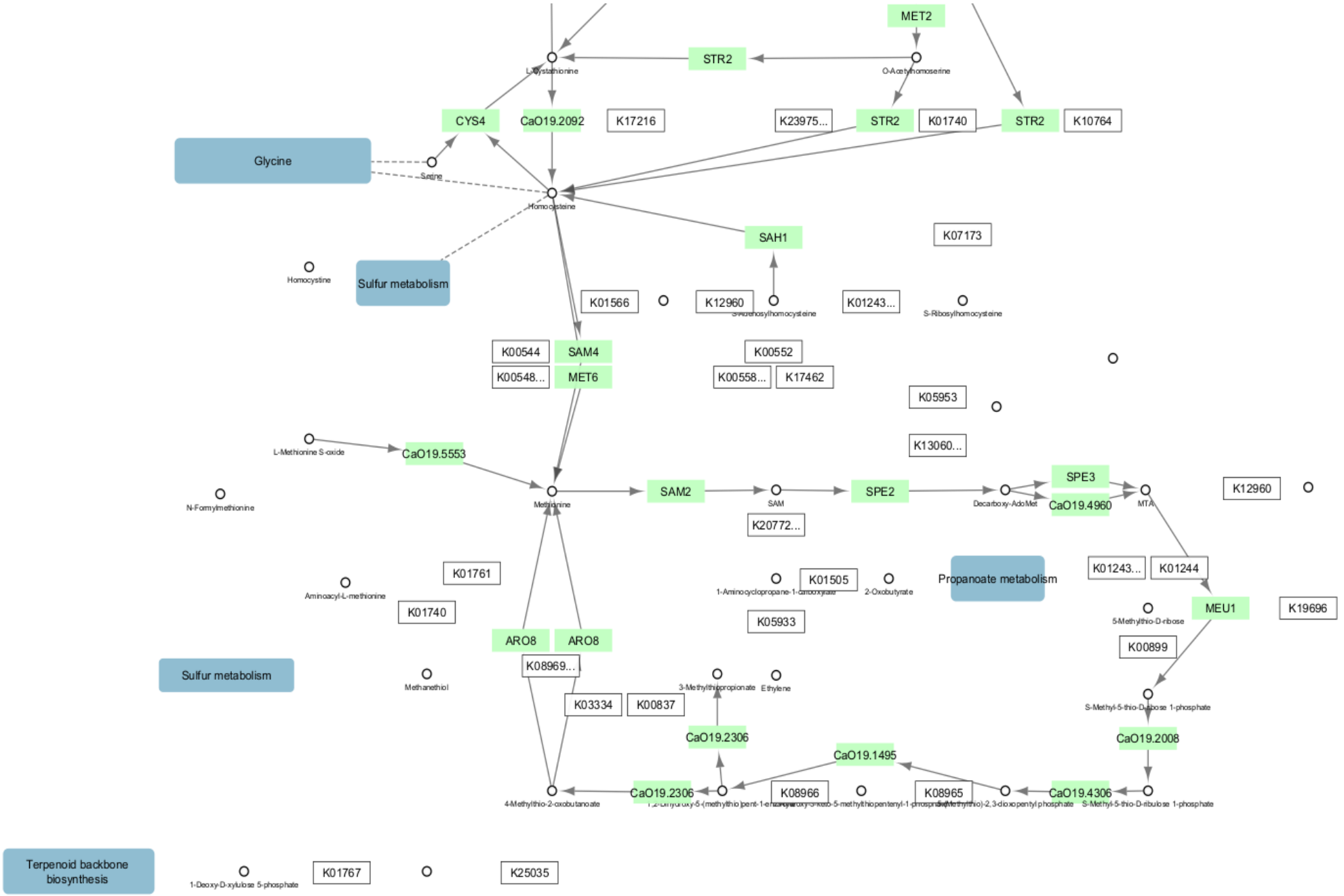
MET2
STR2
L-Cystathionine
O-Acetylhomoserine
CYS4
CaO19.2092
K17216
K23975...
STR2
K01740
STR2
K10764
Glycine
Serine
Homocysteine
SAH1
K07173
Homocystine
Sulfur metabolism
K01566
K12960
S-Adenosylhomocysteine
K01243...
S-Ribosylhomocysteine
K00544
SAM4
K00548...
MET6
K00552
K00558...
K17462
K05953
K13060...
L-Methionine S-oxide
CaO19.5553
N-Formylmethionine
Methionine
SAM2
SAM
SPE2
Decarboxy-AdoMet
SPE3
CaO19.4960
MTA
K12960
K20772...
K01761
Aminoacyl-L-methionine
K01740
1-Aminocyclopropane-1-carboxylate
K01505
2-Oxobutyrate
K05933
Propanoate metabolism
K01243...
K01244
MEU1
K19696
5-Methylthio-D-ribose
K00899
ARO8
ARO8
K08969...
Sulfur metabolism
Methanethiol
3-Methylthiopropionate
Ethylene
K03334
K00837
S-Methyl-5-thio-D-ribose 1-phosphate
CaO19.2008
CaO19.2306
CaO19.1495
CaO19.2306
K08966
K08965
CaO19.4306
4-Methylthio-2-oxobutanoate
S-Methyl-5-thio-D-ribulose 1-phosphate
Terpenoid backbone biosynthesis
1-Deoxy-D-xylulose 5-phosphate
K01767
K25035

Figure 4.7. KEGG pathway map of cysteine methionine pathway. Mappable enzymes within the pathway are highlighted in green and were identified with the aid of the following database https://www.kegg.jp/pathway/map=cal00270&keyword= through http://www.cytoscape

4.4 Conclusion

In conclusion, the proteomic analysis of *Candida albicans* under the influence of dosage dependent lethal mutants of ubiquitin, namely UbEP42, UbL50P and UbI61T revealed the downregulation of key proteins involved in metabolism, stress response, bringing about alterations in the expression of proteins associated with cell cycle regulation, stress tolerance, viability, ubiquitin chain formation, cell wall synthesis, protein quality control, and virulence factor secretion. These molecular-level events shed light on the molecular mechanisms underlying the phenotypic alterations observed in *C. albicans*. Further elucidation on the interplay of the already known proteins and finding the roles of the proteins unidentified so far will contribute to our understanding of fungal ubiquitin signaling and its implications for *C. albicans* biology and virulence. These findings provide valuable insights into the consequences of ubiquitin dysregulation and their impact on the molecular makeup underlying the pathogenicity of *C. albicans.*