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REGULATION OF RAS-INDUCED HYPHAL FORMATION BY HISTONE ACETYLASE GCN5 IN *CANDIDA ALBICANS*

Lalremruata Hauhnar * and H. Lalhruaitluanga

Department of Biotechnology, Mizoram University, Aizawl - 796004, Mizoram, India.

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Correspondence to Author:
Dr. Lalremruata Hauhnar

DST Young Scientist, Department of Biotechnology, Mizoram University, Aizawl - 796004, Mizoram, India.

E-mail: teteahauhnar@gmail.com

ABSTRACT: *Candida albicans* is the leading cause of superficial and systemic fungal infections in human beings and is very common in people with a weakened immune system. It can grow either as budding yeast, a pseudohyphae form, or in a true hyphal form, and the ability to switch between these three forms is important for its pathogenicity. To date, an in-depth study on the relationship between histone modification and hyphal formation is limited, and our study has therefore aimed at adding further information regarding this relationship. In our study, we have found that by disrupting the gene *GCN5*that codes for a histone acetylating protein Gcn5, there was indeed a defect in the hyphal formation confirming the previous report made by Chang *et al.,* (2015). We also found that the defect in the hyphal formation was probably due to regulation of hyphal inducing signaling protein Ras1 by Gcn5 leading to its reduced expression as well as its downstream target hyphal transcription factors Efg1 and Cph1 in the homozygous mutant. The reduced expression of Ras1 is probably caused by the reduced binding of RNA pol II onto the promoter of *RAS1*.

INTRODUCTION: *C. albicans* is a dimorphic fungus that either grows as yeast or in a filamentous form that causes oral and genital infections in human beings. Almost all of us carry it in our gastrointestinal and genitourinary tracts, and to a lesser extent in our skin. *Candida* infections are a common occurrence in people with weak immune systems, for *e.g.*, in patients who undergo cancer chemotherapy treatment or are HIV infected, or when the competing flora is eliminated after antibiotic treatment [1]. The organism can grow either as budding yeast, or in pseudohyphal or hyphal forms in response to environmental cues and can cause severe infections in immuno-compromised individuals [1].

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This change in morphology is an important factor for the virulence of *C. albicans* and its survival inside the host [2]. The hyphae are long tube-like filaments with no constrictions at the site of septation, while pseudohyphae have constrictions and are wider than hyphae [1]. The hyphal form is necessary for the penetration of tissues and escape from the immune system while the yeast form is necessary for the spread of infection in the bloodstream and the pseudohyphae are suggested to be important as a means for the immobile yeast cells to forage for nutrients [2, 3].

Examination of patients with mucosal infections shows that only hyphal forms are found in epithelial cells [1]. Therefore, hyphal formation is important for virulence and mutants; a defect in hyphal induction are generally shown to be avirulent in systemic infections [3, 4]. Several genes are expressed with the induction of hyphal formation such as HWP1, ALS3, RBT5, *etc*. that encode cell wall proteins that help in attachment to

the host as well as in the acquisition of iron from the host cell [5, 6].

Hyphal induction is controlled by several transcription factors such as EFG1 and FLO8 that act downstream of the cAMP-PKA pathway and are positive regulators of hyphal specific genes [1, 7, 8]. Other transcription factors include Cph1 that act downstream of a mitogen-activated protein kinase (MAPK) pathway, Cph2, Tec1, Czf2, Rim101 and Ndt80 [1]. Efg1 seems to be the main regulator of hyphal formation under most conditions as it induces hyphal formation in response to serum, CO_2, neutral pH, GlcNAc in liquid and solid media [1, 9]. The transcription factor Cph1 that acts down-stream of MAPK pathway is also required for the hyphal formation on solid spider medium but not in liquid media [10]. Both cAMP-PKA and MAPK pathways are positively regulated by Ras1 [1, 10].

The genome of eukaryotic cell is highly compacted and organized into a chromatin by histone and non-histone proteins. The smallest unit of chromatin is a nucleosome that consists of an octamer of histone proteins H2A, H2B, H3 and H4 with 147~ bp of DNA wrapped around the core histone proteins [11-14]. Due to the compact organization of the chromatin, the promoters and genes are inaccessible for transcription factors to activate a specific gene [15]. Therefore, in order to access the genome, the chromatin has to undergo a modification, and this occurs very frequently in eukaryotes. The histone proteins composed of two regions, the globular domain and the N-terminal tail [12, 15].

The histone tails undergo two types of modification: ATP-dependent modification and ATP-independent modification [16]. The ATP-independent chromatin remodeling modified the histone tails without hydrolyzing ATP, and modify the histones by acetylation, methylation, phosphorylation, ubiquitination, sumoylation, *etc*. [13] Histone acetylation is the first modification to be discovered, and results in the expression of a gene. The enzymes that catalyzed the reaction are called histone acetylatransferases (HATs) and results in transfer of an acetyl group to a lysine residue of the histone terminal tails [17].

HATs are diverse group of enzymes that can be divided based on their catalytic enzymes. There are five families that include GNAT family, MYST family, SRC family, CBP/p300 family, and $TAF_{II}250$ family [13, 18]. Among the different families of HATs, the GNAT family of HAT is identified by their sequence and structural similarity to Gcn5, the main catalytic subunit of the enzyme complex. In yeast, Gcn5 is required for the progression from G2 to M phase of cell cycle and for mitotic gene expression [19, 20]. They are also involved in transcription activation in an organism [21]. The SAGA complex acetylates histone H3 at the promoter region after its recruitment so that transcription factors could bind to the promoter [22]. GNATs also have a role in DNA repair. In yeast, a member of GNAT family Hat1 is shown to be involved in DNA double-strand break repair. Mutants of Hat1 showed an increase in sensitivity to DNA damaging agents, giving an idea that Hat1 might affect repair at the chromatin assembly level [23].

The previous report made by Chang *et al*., shows that Gcn5 is important for hyphal formation of *C. albicans* and its invasion into epithelial tissues. The report also shows that the deletion of GCN5 leads to impaired hyphal elongation in sensing serum [24].

In our study, we have confirmed that Gcn5 is important for the induction of hyphal formation in *C. albicans* and GCN5 homozygous mutants showed delayed hyphal formation as reported by Chang *et al.* [24] Our study further shows that Gcn5 controls the expression of hyphal transcription factors Efg1 and Cph1 and their upstream regulator Ras1 and that the reduced expression of RAS1 is probably caused by the reduced binding of RNA pol II onto the promoter of RAS1 thus leading to the abnormal hyphal formation in GCN5 deleted strains.

MATERIALS AND METHODS:

Chemicals: All chemicals and reagents were purchased from Merck (India), Sigma Aldrich, Thermo Fisher Scientific, HiMedia (India), and SRL (India) unless otherwise stated.

Construction of Disruption Strains in *C. albicans*: The two alleles of the GCN5 gene were knocked-out by using a PCR-mediated approach. HIS1 marker containing plasmid was amplified with flanking primers (HD FP and HD RP) listed in

Table 1, containing ~60 nucleotides homologous to the start and end of the gene. A wild type BWP17 strain, a kind gift from Dr. Aaron Mitchel, Department of Biological Sciences, Carnegie Mellon University, was transformed with this amplification product using the lithium acetate method; transformants were selected on synthetic defined (SD) medium [2% glucose, 0.67% yeast nitrogen base and 0.2% amino acid mix with the relevant amino acids left out for the appropriate experiments] lacking histidine. The homozygous null strain was constructed from the heterozygous mutant using the same method where the ARG4 gene was amplified along with fragments of the gene using the primers AD FP and AD RP, transformed and plated on SD media lacking arginine and histidine.

TABLE 1: LIST OF PRIMERS USED IN THE STUDY

Name of Primer	Primer Sequence 5'-3'
HD FP	ATGGTTGACAGAAA AAGAACTGCAGCAATACGTGCCGAG
	GATGATGATGAAGAAAATGACCGGGGATCCTGGAGGATGAG
HD RP	CTATACAAAACTACAGTCTTTCAATTTATTATTCATAAACT
	TTTCTAGTTTATTTGCGTTCGGAATATTTATGAGAAACT
AD FP	ATGGTTGACAGAAAAAGAACTGCAGCAATACGTGCCGAG
	GATGATGATGAAGAAAATGACTGTGGAATTGTGAGCGGAAG
AD RP	CTATACAAAACTACAGTCTTTCAATTTATTATTCATAAAC
	TTTTCTAGTTTATTTGCGTTTTTTCCCAGTCACGACGTT
RT GAPDH FP	CAGCTATCAAGAAAGCTTCTG
RT GAPDH RP	GATGAGTAGCTTGAACCCAA
RT EFG1 FP	ACTGCTGGTACCCCTCAAGGT
RT EFG1 RP	ACCTGGTTGTGATGCAGGTGT
RT CPH1 FP	ATGGTGCTATGATTGGCATGA
RT CPH1 RP	CTGCTGTTGTTGTTGTTGTTG
RT RAS1 FP	TACTAGTGCTGTTAATGGTGG
RT RAS1 RP	TGATTGATTTGGAAGATTTGC
Chip RAS1 FP	CGTTTATAGGGTTAATGAATGA
Chip RAS1 RP	AGACGAGATATGAAAAGAGGA

Genomic DNA Isolation from *C. albicans*: An overnight 10 ml culture was harvested, and the pellet resuspended in 500 µl of lysis buffer (100 mM Tris-Cl pH 8.0, 50 mM EDTA, 1% SDS). An acid-washed glass bead of around 0.7 g was added and vortexed for 10 times in pulse (1 min vortex – 1 min ice). The liquid phase was recovered in an Eppendorf tube, and 275 µl of 7 M ammonium acetate (pH 7.0) added, incubated at 65 °C for 5 min, and then incubated on ice for 5 min again. After that, 500 µl of chloroform was added to the mixture, centrifuged for 10 min at 10,000 rpm, then the clear solution took into a fresh tube, and RNase treatment was given for 60 min at 37 °C. 500µl of chloroform was added again, centrifuged for 10 min at 10,000 rpm, the upper portion is taken into a fresh tube, and 1 ml of isopropanol was added to precipitate DNA and incubated at room temperature for 30 min. The precipitated DNA was recovered by centrifuging for 10 min at 10,000 rpm. The pellet washed with 70% ethanol for three times and resuspended in 40 µl of distilled water. The genomic DNA was stored at 4 °C for further use.

Transformation of *C. albicans*: Transformations were performed using lithium-acetate method [25].

Resistance profiling of mutants by spot tests: Cells of *C. albicans* were grown for 24 h in YEPD media at 30 °C in an incubator shaker. Then 2% (v/v) of the cultures were inoculated into fresh media and allowed to grow for 5 h. Cells were resuspended in saline (0.9% NaCl) and adjust the O.D. 600 to 0.2 and five-fold serial dilutions made. 5 µl of cell suspensions from each dilution were spotted on to the SD minimal media plates in the absence and presence of test compounds. The plates were incubated at 30 °C for 16 h.

Hyphal Induction: The *C. albicans* strains were grown overnight at 30 °C in YEPD media for primary cultures, and the secondary cultures were grown in YEPD media containing 10% serum and Spider media at 37 °C using 1% of the primary cultures for 3 h. Then the cells were analyzed for hyphal formation under an Olympus BX41 microscope fitted with Olympus E330 camera, and the image was taken at 40X.

Isolation of RNA from *C. albicans*: 10 ml culture was grown for 16 hrs at 30 °C and harvested using a centrifuge at 5000 rpm for 5 min. The pellet was washed twice using DEPC-water, and 1 ml of Tri-reagent was added for every 0.3 g of the pellet along with 0.3 g of glass beads for every 1 ml of Tri-reagent. The cells were lysed by vortexing 3 times in pulse (1 min vortex – 1 min ice) and then incubated on ice for 5 min. 200 µl of chloroform was added for each 1 ml of Tri-reagent used, the solution vortexed for 15 sec and incubated at room temperature for 10-15 min and centrifuged at 12,000 rpm for 15 min at 4 °C. The upper colorless aqueous phase was transferred to another tube, and 0.5 ml of isopropanol was added for each 1 ml of Tri-reagent used to precipitate RNA. The solution was mixed by vortexing for 15 sec and incubated at room temperature for 5-10 min. The precipitated RNA was centrifuged at 12,000 rpm for 10 min at 4 °C. The supernatant was discarded carefully without disturbing the pellet, and the pellet was washed again with 75% ethanol. After washing, the pellet was dried for 5 min, resuspended in 40 µl of DEPC-water, and stored at -20 °C for further use.

Preparation of Complimentary DNA (cDNA): 2 µg of total RNA was taken and 1 µl of random hexamer primer was added, and the volume of the reaction mixture was made up to 9 µl. The reaction was incubated at 65 °C for 10 min, after that it was chilled on ice as quickly as possible. Then the following were added: RNase inhibitor - 1 µl, 0.1M DTT - 1 µl, 5X reverse transcriptase buffer - 4 µl, 10 mM dNTP - 2 µl, reverse transcriptase – 0.5 µl, and finally DEPC-water - 1.5 µl. After adding everything, the reaction was incubated at 37 °C for 60 min, then incubated at 94 °C for 90 sec to inactivate the reverse transcriptase. The prepared cDNA was stored at -20 °C for future use.

Quantitating Transcript Level: SYBR Green reagent was used to quantitate the transcript levels in wild type and mutant cells. SYBR Green is an asymmetrical cyanine dye that attaches only to double-stranded DNA, and the fluorescence emission of the DNA-dye complex is used to quantify the amount of DNA being produced. The primers used for the study are listed in **Table 1**. For calculating the relative level of transcription, the comparative Ct method of Wong and Medrano [26] was used. The reaction mixture consists of 1X SYBR Green, 0.1 µM each of the forward primer and the reverse primer, and 1 µl of the main cDNA stock.

Chromatin Immunoprecipitation Assay: ChIP protocols for yeast strain from Abcam were used using RNA pol II antibodies from Santa Cruz Biotechnology [27]. The input sample was prepared in the same way without the addition of any antibodies. The samples were analyzed by real-time PCR, and the primers used for the study have been listed in **Table 1**.

RESULTS:

Creation of Knocked-Out Mutants: *Candida albicans* wild type strain BWP17 was obtained from Dr. Aaron Mitchell, Department of Biological Sciences, Carnegie Mellon University. The wild type strain was used for making the heterozygous mutant (G1.1) and homozygous null mutant (G1.2) of GCN5. The mutants were created using homologous recombination of an amplified product of the selection marker with a short stretch of the gene that replaces the original copy of the genome. The selection marker gene, along with a short stretch of the gene GCN5 was amplified using PCR and transformed into the wild type BWP17, as shown in **Fig. 1**.

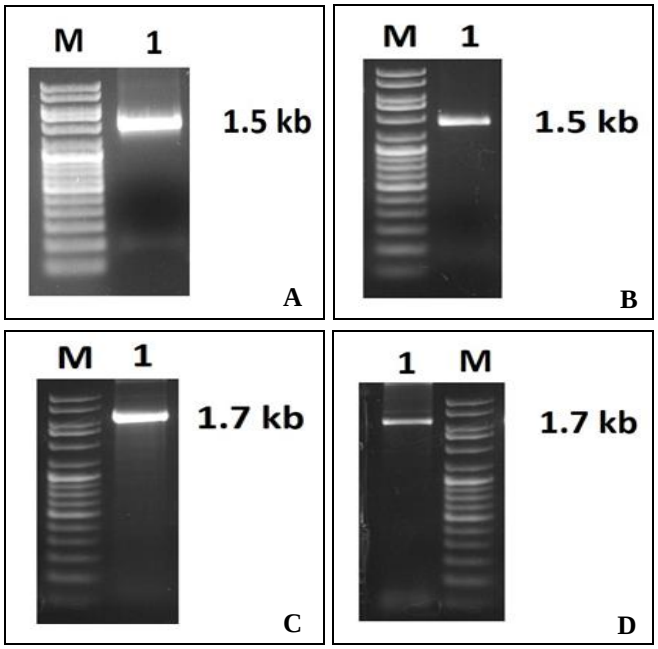

FIG. 1: M – MARKER LANE 1 – SAMPLE LANE A) PCR AMPLIFICATION OF GCN5-HIS1 B) CONFIRMATION OF INTEGRATION OF GCN5-HIS1 IN G1.1 BY PCR C) PCR AMPLIFICATION OF GCN5-ARG4 D) CONFIRMATION OF INTEGRATION OF GCN5-ARG4 IN G1.2 BY PCR

HIS1 gene marker was first used for creating the heterozygous mutant, G1.1, and ARG4 marker was subsequently used for making the homozygous mutant, G1.2. The mutants were confirmed using the primers for the marker gene, *i.e.*, HIS1 and ARG4.

Analysis of Cell Wall Integrity: The mutants were checked against the wild type to see the effect of the CaGCN5 deletion on the cell wall integrity. The mutants showed no changes when treated with congo red. But the treatment of both the mutants with SDS showed resistance compared to the wild type BWP17, as shown in **Fig. 2**. Congo red is a dye that interferes with the construction and stress response of the cell wall, while SDS is a very good agent to check the integrity of the cell wall [28, 29].

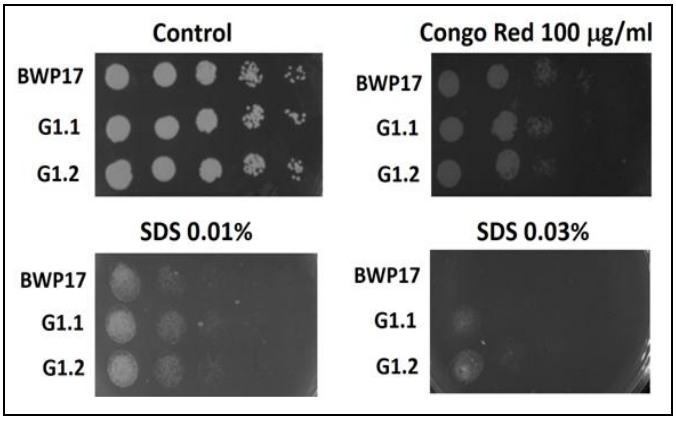

FIG. 2: TREATMENT OF GCN5 MUTANTS WITH CONGO RED AND SDS. SECONDARY CULTURES GROWN FOR 5 h WERE SUSPENDED IN NORMAL SALINE AND THE OD WAS ADJUSTED TO 0.2 AT 600 nm. 5 µL OF THE SERIALLY DILUTED CULTURES WAS SPOTTED ONTO SD MINIMAL MEDIA PLATES IN THE ABSENCE AND PRESENCE OF TEST COMPOUNDS

Defect in Hyphal Formation on CaGCN5 Mutants: Hyphal formation is an important step for the pathogenesis of *Candida albicans*. The knocked-out mutants of CaGCN5 were checked for their ability to form hyphal formation against the wild type BWP17. As shown in **Fig. 3**, three different types of cells were found, namely hyphae, pseudohyphae, and yeast type of cells, after growing them in liquid spider media. The wild type BWP17 and heterozygous mutant G1.1 showed a high degree of hyphal formation, and the percentage of cells showing hyphae was very high. But the result was completely opposite in the homozygous mutant G1.2, where the number of cells showing hyphae form was very less while the yeast form was very common.

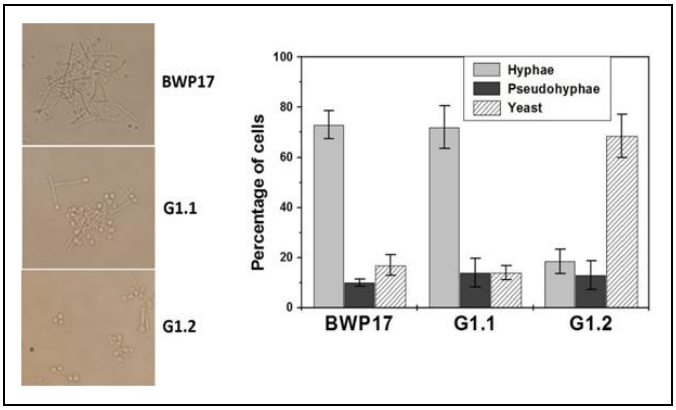

FIG. 3: HYPHAL INDUCTION OF GCN5 MUTANTS IN SPIDER MEDIA. SECONDARY CULTURES WERE GROWN IN SPIDER MEDIA AT 37 °C USING 1% OF THE PRIMARY CULTURES FOR 3 h. THE IMAGES WERE TAKEN AT 40X USING OLYMPUS BX41 MICROSCOPE FITTED WITH OLYMPUS E330 CAMERA.

The hyphal formation was also checked in another hyphal inducing agent fetal bovine serum (10% FBS + YEPD). The same result was also found in these media where there was a high number of cells showing hyphae form in BWP17 and the heterozygous mutant G1.1 while the number of cells showing yeast form was very less. But the homozygous mutant G1.2 showed a very low percentage of cells showing hyphal form while the yeast form was very high, as shown in **Fig. 4**.

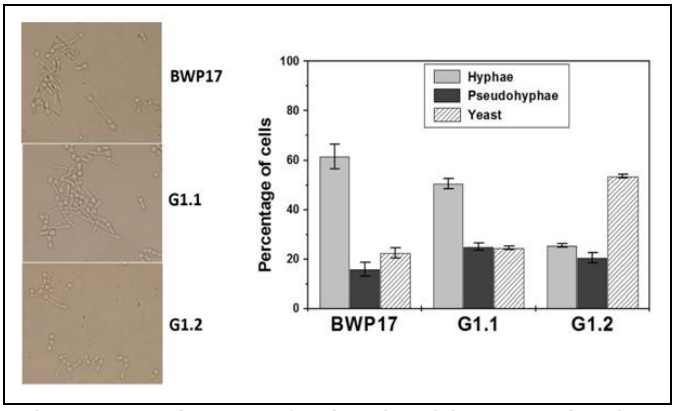

FIG. 4: HYPHAL INDUCTION OF GCN5 MUTANTS IN FETAL BOVINE SERUM. SECONDARY CULTURES WERE GROWN IN YEPD MEDIA CONTAINING 10% OF FBS AT 37 °C USING 1% OF THE PRIMARY CULTURES FOR 3 h. THE IMAGES WERE TAKEN AT 40X USING OLYMPUS BX41 MICROSCOPE FITTED WITH OLYMPUS E330 CAMERA

Effect of Hyphal Transcription Factors on CaGCN5 Mutants: As mentioned before, the hyphal induction in *C. albicans* is controlled by several transcription factors that promote the conversion of yeast to hyphae form. Of the mentioned transcription factors, the expression level of two transcription factors Efg1 and Cph1

was checked by real-time PCR, and it was found that their transcript level was both down by around 50% in the homozygous mutant G1.2 as compared to the wild type BWP17 as shown in **Fig. 5**.

As mentioned above, both the transcription factors Efg1 and Cph1 are controlled by Ras1 through the cAMP-PKA and MAPK pathway, respectively. The level of RAS1 gene expression was checked, and the result was that it was down by almost 50% in the homozygous mutant G1.2 compared to the wild type BWP17, as shown in **Fig. 5**.

The heterozygous mutant G1.1 was not checked for the expression of hyphal transcription factors as it did not show any significant changes in hyphal induction when compared to the wild type.

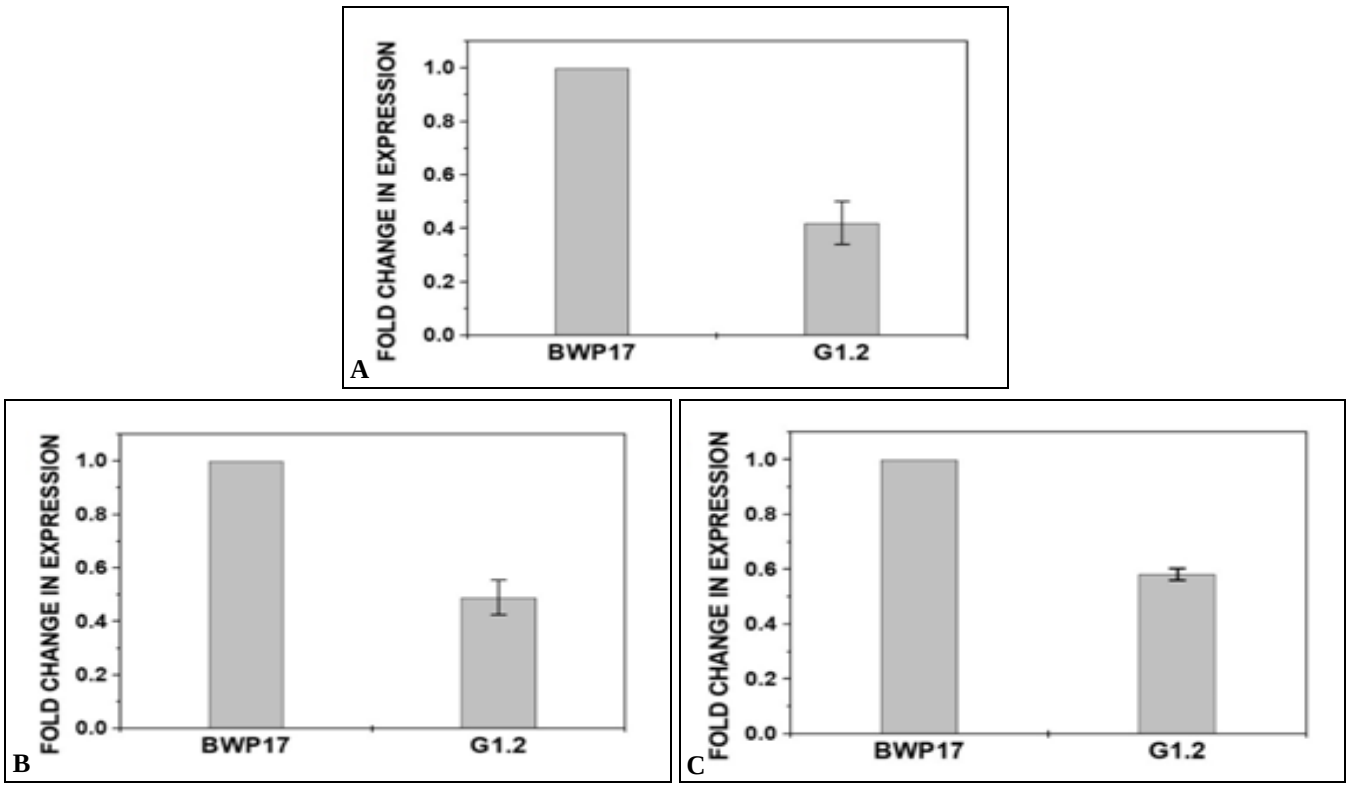

FIG. 5: TRANSCRIPT LEVEL OF HYPHAL TRANSCRIPTION FACTORS A) EFG1 B) CPH1 AND C) RAS1 GENES IN GCN5 NULL MUTANTS. cDNA WAS MADE FOR EACH STRAIN AND THE LEVEL OF EACH TRANSCRIPT WAS CHECKED BY GENE SPECIFIC PRIMER USING APPLIED BIOSYSTEM 7500 FAST REAL-TIME PCR SYSTEM

Reduced RNA pol II Occupancy on the RAS1 Promoter: The reduction in the RAS1 expression could be due to many reasons like the absence of the RNA polymerase II, the general transcription factors and activators, and enhancers. Out of this, RNA polymerase II is the actual protein that synthesizes mRNA from DNA [30, 31]. An antibody against RNA polymerase II was used for chromatin immunoprecipitation (ChIP) experiment to determine the level of RNA polymerase II at the RAS1 promoter in the wild type and the homozygous mutant. It was found that there was indeed a decrease of around 65% enrichment in the homozygous mutant G1.2 when compared to the wild type BWP17, as shown in **Fig. 6**. The decrease in the fold enrichment of RNA polymerase II on the RAS1 promoter could be one of the reasons for the lowered expression of Ras1, leading to reduced hyphal formation in the homozygous mutant G1.2.

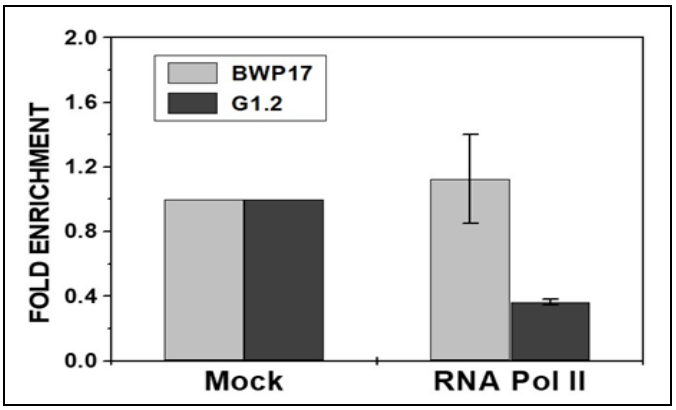

FIG. 6: RNA POLYMERASE II OCCUPANCY AT RAS1 PROMOTER. FOLD ENRICHMENT WAS CALCULATED WITH RESPECT TO THE MOCK ChIP DONE USING NO ANTIBODY CONTROL

DISCUSSION: The genome of a eukaryote is highly compacted due to the binding of histone proteins. The histone binding must be loosened for DNA binding proteins to access the genome for processes such as DNA replication, gene expression, DNA repair, *etc*. One way of achieving this is by acetylation of histones to loosen the chromatin and Gcn5 is one of the enzymes that is involved in the acetylation of histone N-terminal tails [32]. The previous study by Chang *et al.,* [24] shows that Gcn5 is involved in the hyphal formation of *C. albicans* and its invasion into a solid media. The mechanism of hyphal formation regulation by Gcn5 has not been properly elucidated. Therefore, to study the regulation of hyphal formation by Gcn5 in *C. albicans*, heterozygous and homozygous null mutants were created using homologous recombination in our study to examine the function of Gcn5 in *C. albicans*.

The ability to form hyphae is very important for the survival of the *C. albicans* as well as for its pathogenicity. In our study, we had found that there was a defect in the hyphal formation when both the copies of GCN5 were disrupted confirming the previous findings by Chang *et al.* [24] The hyphal formation was checked in two different types of liquid media, namely spider media and YEPD media with fetal bovine serum. The homozygous null mutant showed a clear defect in hyphal formation with the two types of media, and around 70% of the cells remained in the yeast form while the wild type showed a very high number of cells in the hyphae form. As for the heterozygous mutant, there was no change at all in the hyphal formation when compared to the wild type, which showed that disruption of one copy of GCN5 is not enough to induce a defect in hyphal formation. The results also showed that being an activator of gene expression, Gcn5 seems to play an important role in the regulation of hyphal formation in *C. albicans.*

Hyphal formation in *C. albicans* is controlled positively by several transcription factors and of these, Efg1 seems to be the most important one as it regulates the induction of hyphal formation under most conditions [1, 10]. In our study, we have found that the levels of EFG1 expression had gone down by more than 50% in the homozygous null mutant while another important transcription factor, Cph1 transcript, levels had gone down by around 50% in the homozygous null mutant that may have resulted in the defect in hyphal formation after the disruption of GCN5 in *C. albicans*. As mentioned earlier, Efg1 and Cph1 are controlled by another important protein Ras1.

Ras1 activates adenylyl cyclase Cyr1 that activates the cAMP-PKA and MAPK pathway that controls the expression of Efg1 and Cph1, respectively, and it had been earlier reported that RAS1 deletion in *C. albicans* results in hypofilamentation [1, 10]. Our study shows that the expression of RAS1 had gone down by around 45% in the homozygous null mutant, which is suggestive that Gcn5 may be controlling the expression of the RAS1 gene. By doing ChIP, we had further shown that the occupancy of RNA polymerase II on the RAS1 promoter had also been affected in the homozygous mutant of GCN5, where RNA pol II level was down by around 60%.

CONCLUSION: Gcn5 is a histone acetyl-transferase enzyme that is an important regulator of positive gene expression as acetylation induces the loosening of histone proteins resulting in the binding of transcription factors onto the promoter [12, 33]. From our study, Gcn5 seems to be controlling the expression of hyphal transcription factors Efg1 and Cph1 by regulating the expression of Ras1. In turn, the expression of Ras1 seems to be controlled by affecting the binding of RNA pol II on the *RAS1* promoter. Therefore, Gcn5 is likely very important for hyphal formation in *C. albicans* by controlling the expression of hyphal transcription factors. Since hyphal formation is important for pathogenicity, targeting the Gcn5 could play an important role in fighting against *C. albicans.*

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CONFLICTS OF INTEREST: The author declares no conflict of interest.

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