Modulation of azole sensitivity and filamentation by *GPI15*, encoding a subunit of the first GPI biosynthetic enzyme, in *Candida albicans*

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Glycosylphosphatidylinositol (GPI)-anchored proteins are important for virulence of many pathogenic organisms including the human fungal pathogen, *Candida albicans*. GPI biosynthesis is initiated by a multi-subunit enzyme, GPI-N-acetylglucosaminyltransferase (GPI-GnT). We showed previously that two GPI-GnT subunits, encoded by CaGPI2 and CaGPI19, are mutually repressive. CaGPI19 also co-regulates CaERG11, the target of azoles while CaGPI2 controls Ras signaling and hyphal morphogenesis. Here, we investigated the role of a third subunit. We show that CaGpi15 is functionally homologous to *Saccharomyces cerevisiae* Gpi15. CaGPI15 is a master activator of CaGPI2 and CaGPI19. Hence, CaGPI15 mutants are azole-sensitive and hypofilamentous. Altering CaGPI19 or CaGPI2 expression in CaGPI15 mutant can elicit alterations in azole sensitivity via CaERG11 expression or hyphal morphogenesis, respectively. Thus, CaGPI2 and CaGPI19 function downstream of CaGPI15. One mode of regulation is via H3 acetylation of the respective GPI-GnT gene promoters by Rtt109. Azole sensitivity of GPI-GnT mutants is also due to decreased H3 acetylation at the CaERG11 promoter by Rtt109. Using double heterozygous mutants, we also show that CaGPI2 and CaGPI19 can independently activate CaGPI15. CaGPI15 mutant is more susceptible to killing by macrophages and epithelial cells and has reduced ability to damage either of these cell lines relative to the wild type strain, suggesting that it is attenuated in virulence.

GPI anchored proteins play important roles as adhesion molecules, enzymes, activation antigens, differentiation markers, and protozoan coat components. The GPI anchor is synthesized in the endoplasmic reticulum of eukaryotes by the concerted action of multiple enzymes in a pathway that is sequential for the most part1. The pathway is essentially conserved in all eukaryotic organisms and generates a glycolipid anchor of the form NH2CH2CH2PO4H-6Manα1-2Manα1-6Manα1-4GlcNα1-6D-myo-inositol-1-HPO4-lipid, where the lipid can be diacylglycerol, alkylacylglycerol or ceramide. Yet, several species-specific variations in the GPI biosynthetic pathway exist1,2.

The process starts with the transfer of N-acetylglucosamine (GlcNAc) from UDP-GlcNAc to phosphatidylinositol (PI), a reaction that is in all probability catalyzed by Gpi3 (yeast) or Pig-A (mammals). With the exception of *Giardia lamblia*, in all other eukaryotic systems, Gpi3/Pig-A is assisted by several other proteins that number anywhere between 3 to 6, depending upon the organism, and which together form the GPI-N-acetylglucosaminyl transferase (GPI-GnT) complex in the endoplasmic reticulum3. In humans, the GPI-GnT complex includes, besides Pig-A, five other subunits, Pig-H, Pig-C, Pig-P, Pig-Q and Pig-Y4. Of these, homologs of Pig-C, Pig-P and Pig-Q are present in all eukaryotes. In yeast, they correspond to Gpi2, Gpi19 and Gpi1, respectively. **PIG-Y** is predicted to be homologous to yeast **ERII**, although the latter could not functionally complement Daudi cells.

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defective in \textit{PIG-Y}. \textit{PIG-H} or yeast \textit{GPI15} is absent in \textit{Caenorhabditis elegans} and \textit{Entamoeba histolytica}. In yeast, deletion of all other than \textit{GPI1} and \textit{ERI1}, are lethal\textsuperscript{94}. \textit{C. albicans} is an opportunistic pathogen that causes severe invasive as well systemic infections in immunocompromised individuals often leading to mortality. GPI anchored proteins in this organism are important for yeast-to-hyphae transition as well as for virulence\textsuperscript{22}. Disrupting the GPI biosynthetic pathway results in lethality\textsuperscript{8,10} suggesting that GPI biosynthesis is essential in the organism. In the first set of reports on the GPI-GnT complex of \textit{C. albicans}, we showed the importance of \textit{CaGPI19} in growth, drug response and hyphal morphogenesis of this organism\textsuperscript{11,12}. The \textit{CaGPI19} deficient mutant was azole sensitive and hyperfilamentous\textsuperscript{11}. A mutual co-regulation existed between \textit{CaGPI19} and \textit{CaERG11}, an important gene in the ergosterol biosynthetic pathway and the target of azoles\textsuperscript{32}.

We then went on to show that a mutant in a second subunit, \textit{CaGPI2}, was azole resistant and hypofilamentous. Further, \textit{CaGPI2} specifically controlled hyphal morphogenesis via Ras signaling. It was also negatively co-regulated with \textit{CaGPI19}\textsuperscript{33}.

In the present manuscript, we have explored the role of a third subunit, \textit{CaGPI15}, in \textit{C. albicans}. We show that \textit{CaGPI15} is important for growth, cell wall integrity and GPI biosynthesis in \textit{C. albicans}. It also affects response to azole drugs as well as hyphal morphogenesis in \textit{C. albicans}. It does so because it simultaneously activates both \textit{CaGPI2} and \textit{CaGPI19} which function downstream of \textit{CaGPI15}. Thus, here too \textit{CaGPI2} controls hyphal morphogenesis via CaRas1 and \textit{CaGPI19} controls sensitivity to azoles by regulating \textit{CaERG11} levels. The downregulation of \textit{CaERG11} in mutants of \textit{CaGPI15} as well as \textit{CaGPI19} occurs due to decrease in \textit{H3} acetylation on the promoter of \textit{CaERG11}. Both \textit{CaGPI2} and \textit{CaGPI19} can also independently activate \textit{CaGPI15} levels.

\section*{Results}

\subsection*{Cloning of \textit{CaGPI15} gene from \textit{C. albicans}}

The putative \textit{CaGPI15} gene was identified using human \textit{PIG-H} gene as the query sequence for BLAST analysis as well as using the information available at Prof. Eisenhaber's website as explained in Materials and Methods. The sequence obtained also compared very well with that reported previously\textsuperscript{14}. The putative \textit{CaGPI15} protein showed roughly 26.23% and 21.94% identity with Gpi15 sequences from \textit{Saccharomyces cerevisiae} and \textit{Saccharomyces pombe}, respectively (Supplementary Fig. 1A). The gene was subsequently cloned from the genomic DNA of \textit{C. albicans} using gene-specific primers.

\subsection*{\textit{CaGPI15} gene complements the \textit{S. cerevisiae} \textit{GPI15} gene}

The \textit{ScGPI15} gene of YPH500 was placed under the control of the \textit{GAL1} promoter. This strain (YPH-pGAL1-ScGPI15) grew well in the presence of galactose but was unable to grow in glucose (Supplementary Fig. 1B). When \textit{CaGPI15} was introduced in this strain (YPH-pGAL1-ScGPI15-CaGPI15), the strain was able to grow in glucose (Supplementary Fig. 1B). Growth complementation was accompanied by restoration of GPI-GnT activity (Supplementary Fig. 1C). Thus, despite low sequence conservation, \textit{CaGPI15} is the functional homolog of \textit{ScGpi15}.

\subsection*{Chromosomal disruption of \textit{CaGPI15} gene}

Heterozygous (\textit{CaGPI15Hz}) and conditional null (\textit{Cagpi15 null}) mutants of \textit{CaGPI15} were generated in the \textit{C. albicans} BWP17 strain using a PCR based approach\textsuperscript{15,16}. \textit{CaGPI15Hz} had one allele of \textit{CaGPI15} disrupted with a \textit{HIS1} nutritional marker\textsuperscript{15}. \textit{Cagpi15 null} strain was made in the \textit{CaGPI15Hz} background with the second \textit{CaGPI15} allele placed under the control of the repressible \textit{MET3} promoter. Since \textit{URA3} is known to alter gene expressions in \textit{S. cerevisiae}\textsuperscript{34}, one copy of \textit{URA3} was inserted at the \textit{RPS1} locus in BWP17 (BWP17\textsuperscript{URA3}) as well as in \textit{CaGPI15Hz} (\textit{CaGPI15Hz-URA3}) and these were used as controls in studies on all mutants that involved use of \textit{URA3} as a selection marker. The downregulation of \textit{CaGPI15} expression levels were confirmed by transcript level analysis (Supplementary Fig. 2A).

\subsection*{Depletion of \textit{CaGpi15} affects growth of \textit{C. albicans}}

The growth of \textit{CaGPI15Hz}, on solid and liquid medium was comparable to that of the wild type strain (Fig. 1A(i,ii)). The \textit{Cagpi15 null} on the other hand, grew slower on solid minimal media containing Met/Cys (Fig. 1A(iii)). Further, in liquid medium, the doubling time for the \textit{Cagpi15 null} in the presence of 10 mM Met/Cys was found to be higher than in the absence of Met/Cys (Fig. 1A(iv); Supplementary Table 2).

\subsection*{Depletion of \textit{CaGpi15} results in reduced GPI-GnT activity in \textit{C. albicans}}

We previously showed that the GPI-GnT activity was reduced in the \textit{CaGPI15Hz} strain\textsuperscript{17}. Not only was the GPI-GnT activity significantly lower in the \textit{CaGPI15Hz} (~50%) with or without the \textit{URA3} marker as compared to the wild type controls it was further reduced in the \textit{Cagpi15 null} strain (28% activity) under repressive conditions of growth (Fig. 1C). Under permissive growth conditions in the \textit{Cagpi15 null} there is no significant decrease in the GPI-GnT activity (47%) as compared to \textit{CaGPI15Hz-URA3} (Fig. 1C). That a drop in GPI-GnT activity of roughly 50% does not seem to cause a corresponding reduction in growth of the strain would suggest that relatively low levels of GPI biosynthetic activity are sufficient for the growth of \textit{C. albicans}. This has also been reported in other GPI biosynthetic mutants\textsuperscript{8,11,12,29}. However, when GPI biosynthesis drops below a certain threshold, as is seen in the conditional null strain under repressive growth conditions, then it affects the growth of the fungus. GPI-GnT activity was restored in \textit{CaGPI15} revertant strain where one allele of \textit{CaGPI15} was reintroduced in the \textit{CaGPI15Hz} using the constitutively active \textit{pACT1} promoter (Fig. 1C).

\subsection*{Depletion of \textit{CaGpi15} causes cell wall defects}

The \textit{CaGpi15} knock-down mutants showed several cell wall defects, including increased clumping when grown to near saturation levels and lower chitin and beta glucan levels in the cell wall versus the wild type strain (Supplementary Fig. 2B,C; Supplementary Table 3). The cell wall defects were reversed in the \textit{CaGPI15} revertant strain (Supplementary Fig. 2B, C(i–iv)), suggesting that the cell wall defects were specifically due to depletion of \textit{CaGpi15}.
CaRas1-dependent signaling pathway is altered in the CaGPI15 mutants. As also shown previously\textsuperscript{17}, hyphal growth of CaGPI15Hz was noticeably lesser than that of the wild type BWP17 strain on solid as well as liquid hyphae-inducing media at 37°C but was restored in the CaGPI15 revertant cells (Fig. 1B(i–iv); Fig. 1D(i–iv); Supplementary Fig. 3A), suggesting that this effect was specific to CaGPI15.

We have previously shown that the Ras/cAMP dependent PKA activity is altered in mutants of the first step of GPI anchor biosynthesis in C. albicans\textsuperscript{12,13,17}. Hyperactive Ras mutants are heat shock sensitive\textsuperscript{13,20} and reduced Ras signaling correlates with heat-shock resistance\textsuperscript{13}. CaGPI15Hz was resistant to heat shock as compared to BWP17 (Fig. 1E(i)), suggesting that Ras-dependent cAMP/PKA signaling was decreased in this mutant.

C. albicans has two Ras proteins, CaRas1 and CaRas2 of which CaRas1 is known to be the major determinant of hyphal growth\textsuperscript{21}. Overexpression of CaRAS1 restores filamentation in CaGPI5Hz while overexpression of CaRAS2 does not (Fig. 1E(ii,iii); Supplementary Fig. 3B).

CaGPI15 mutant strains are sensitive to azoles due to compromised ergosterol biosynthesis. CaGPI15Hz as well as Cagpi15 null cells were sensitive to azoles as compared to controls (Fig. 2A(i,ii); Supplementary Fig. 4A(i,ii)). This sensitivity was reversed in the CaGPI15 revertant strain (Fig. 2A(iii); Supplementary Fig. 4A(iii)). Azoles target CaErg11, the lanosterol demethylase in the ergosterol biosynthetic pathway of C. albicans\textsuperscript{22}. Hence, the levels of CaERG11 transcripts in cells of CaGPI15Hz and Cagpi15 null were examined. CaERG11 levels were significantly reduced in both cases (Supplementary Fig. 4B(ii)). CaERG11 levels were restored in the CaGPI15 revertant strain (Supplementary Fig. 4B(ii)). The reduction in CaERG11 levels...
correlated well with the accumulation of lanosterol, the substrate of CaErg11, and a reduction in total ergosterol levels in the CaGPI15Hz and CaGPI15 null strains (Fig. 2).

Upc2 is a transcription factor that controls CaERG11 levels in the cell. The occupancy of both RNA Pol II (RNAPII) as well as Upc2 were significantly reduced on the promoter of CaERG11 in CaGPI15Hz-URA3 as compared to BWP17URA3 when probed using primer pair 2 (Fig. 2C(i,ii)), suggesting that the promoter had reduced accessibility for transcription. This also correlated well with the fact that acetylation of histone H3, as assessed using the H3K56 antibody, was significantly lower on the promoter of CaERG11 in CaGPI15Hz-URA3 as compared to that in BWP17URA3 (Fig. 2C(ii) & Supplementary Fig. 4D).

The role of histone acetylation in the regulation of CaERG11 expression in C. albicans was further investigated. H3K56 and H3K9 acetylation are mediated by Rtt109, a histone acetyltransferase. As can be seen from (Fig. 2D(i,ii); Supplementary Fig. 5D), the expression of CaERG11 is restored in CaGPI15Hz/pACT1-RTT109 strain. The transcript levels of both RTT109 and CaERG11 were monitored in the mutants relative to BWP17URA3. (ii) The H3K56Ac levels in whole cell lysate were estimated in the mutants relative to BWP17URA3. H3 levels were taken as a loading control. A cropped representative image is shown, and the full-length image is displayed in Supplementary Fig. 5D (iii) The CaERG11 promoter occupancy of RNA pol II, H3K56Ac and H3K9Ac in the mutants relative to BWP17URA3. (E) Azole sensitivity was reversed in CaGPI15Hz/pACT1-RTT109 strain. Growth curve analysis of CaGPI15Hz/pACT1-RTT109 mutant in 0.2μg/ml ketoconazole. The doubling time of CaGPI15Hz/pACT1-RTT109 in the presence of ketoconazole is almost similar with the wildtype strain grown in the same condition (Supplementary Table 2). (F) CaGPI15Hz/pACT1-RTT109 strain accumulates ergosterol. Relative quantification of ergosterol was done using GC-MS. P-values for CaGPI15Hz were calculated relative to the wild type while for CaGPI15Hz/pACT1-RTT109 it was relative to CaGPI15Hz-URA3.
significantly altered in the \textit{CaGPI15Hz} strain. \textit{RTT109} was then expressed under the control of \textit{pACT1} promoter in the \textit{CaGPI15Hz} strain and its overexpression was confirmed (Fig. 2D(i)). \textit{CaERG11} transcript levels were upregulated in these cells (Fig. 2D(i)). ChIP analysis showed that overexpression of \textit{RTT109} in \textit{CaGPI15Hz} cells increased acetylation of H3, as assessed by anti-H3K56 or anti-H3K9 antibodies, and increased the occupancy of RNAPII on the \textit{CaERG11} promoter (Fig. 2D(iii)) Overexpression of \textit{RTT109} in \textit{CaGPI15Hz} cells also reversed the azole sensitivity (Fig. 2E; Supplementary Fig. 4C) and restored ergosterol levels in these cells (Fig. 2F), confirming that acetylation of H3 by Rtt109 regulates the expression of \textit{CaERG11} in this strain.

Separately, in order to ensure that the reduced H3 acetylation was not due to a global reduction in acetylation or due to defects in nucleosome assembly, we checked the H3 acetylation levels at two different intergenic regions (Chromosome 5 and Chromosome R) in BWP17\textit{URA3}, \textit{CaGPI15Hz-URA3} and \textit{CaGPI15Hz-pACT1-RTT109} by ChIP (Supplementary Fig. 4E(i,ii). No reduction in H3 acetylation was observed in the two strains at these positions relative to the control, BWP17\textit{URA3}, suggesting that the ChIP signal observed is not a nucleosome assembly dependent effect and was instead due to a reduction in acetylation levels at the promoter of \textit{CaERG11}.

The acetylation mediated by Rtt109 is catalyzed with two histone chaperones, Vps75 and Asf1 \cite{26}. While the latter has been shown to be important for H3K56Ac alone, the former has been shown to be important for acetylation of H3 at K9 as well K56\cite{25}. A \textit{VPS75Hz} mutant should, therefore, mimic a strain defective in acetylation by Rtt109. To confirm that acetylation of H3 at the \textit{CaERG11} promoter was important for its regulation in \textit{C. albicans}, ChIP analysis was done in a \textit{VPS75Hz} strain generated in the lab. The acetylation of H3 dropped to a significant extent at the promoters of \textit{CaERG11}, \textit{CaGPI15}, \textit{CaGPI2} and \textit{CaGPI19} in this strain when probed using anti-H3K56 antibody (Supplementary Fig. 4F(i–iv)).

\textit{CaGPI2} and \textit{CaGPI19} levels in the \textit{CaGPI15} heterozygous strain can also be restored by \textit{RTT109} overexpression. Transcript levels of both \textit{CaGPI2} and \textit{CaGPI19} were found to be reduced in \textit{CaGPI15Hz} as compared to BWP17 (Fig. 3A(i)) and were restored in the \textit{CaGPI15} revertant strain (Fig. 3A(ii)), suggesting that this effect was specifically linked to \textit{CaGPI15} levels. It should be noted that the expression of two housekeeping genes (\textit{CaUBC13}, \textit{CaACT1}), two ergosterol biosynthesis genes downstream to \textit{CaERG11} (\textit{CaERG3}, \textit{CaERG4}) and

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\caption{\textit{CaGPI2} and \textit{CaGPI19} are downstream of \textit{CaGPI15}. (A) Transcript levels of \textit{CaGPI2} and \textit{CaGPI19} are controlled by \textit{CaGPI15}. (i) The transcript levels of both \textit{CaGPI2} and \textit{CaGPI19} in \textit{CaGPI15Hz} as compared to BWP17. (ii) The transcript levels of \textit{CaGPI15}, \textit{CaGPI2} and \textit{CaGPI19} in the \textit{CaGPI15} revertant. P-values were calculated with respect to the wild type controls. (B) H3K56Ac and transcript levels of \textit{CaGPI2}, \textit{CaGPI15} and \textit{CaGPI19} are controlled by Rtt109. (i) Schematic representation of the promoters of \textit{CaGPI15}, \textit{CaGPI2} and \textit{CaGPI19} along with their H3K56Ac positions. (ii) The relative H3K56Ac levels at the promoters of \textit{CaGPI15}, \textit{CaGPI2} and \textit{CaGPI19} in the indicated strains versus wild type. (iii) Transcript levels of \textit{CaGPI15}, \textit{CaGPI19} and \textit{CaGPI2} upon \textit{RTT109} overexpression in the \textit{CaGPI15Hz}. (C) Hyphal morphogenesis is restored upon overexpression of \textit{RTT109}. (i) and (ii) The hyphal growth and quantification of hyphal growth in various strains for up to 60 minutes in liquid spider media at 37 °C. A minimum of 100 cells were used for the statistical analysis. The experiment was repeated twice in duplicates; averages with standard deviations are shown.}
\end{figure}
levels. (i) Strains overexpressing were grown in liquid SD medium containing 0.2 CaGPI19. The azole response of the strains correlates with Ca to either BWP17 or BWP17URA3. (ii) Expression was monitored in the mutants strains relative to BWP17URA3. (iii) Response to azoles was monitored in using GC-MS. P-values for all heterozygous mutants were calculated with respect to the CaGPI19 mutant. All strains were grown with or without 0.2 µg/ml ketoconazole. Supplementary Table 2 contains the doubling time for the mutants both in the presence/absence of ketoconazole. Two independent experiments in duplicates were done for confirmation. (D) Rtt109 regulates CaERG11 transcription and sensitivity to azoles in the CaGPI19 mutant. (i) ChIP analysis was done to compare the H3K56Ac of CaERG11 promoter in the mutant strains relative to BWP17URA3. (ii) Transcript levels of RTT109 and CaERG11 were monitored in the mutant strains relative to BWP17URA3. (iii) Response to azoles was monitored in CaGPI19Hz/pACT1-RTT109 mutant. All strains were grown with or without 0.2 µg/ml ketoconazole and the doubling time was calculated (Supplementary Table 2). The experiments were repeated twice in duplicates. (iv) Relative quantification of ergosterol was done in different mutants of CaGPI19 using GC-MS. P-values for all heterozygous mutants were calculated with respect to the wild type controls while that for overexpression mutants are with respect to CaGPI19Hz-URA3.

Figure 4. (A) Hyphal morphogenesis correlates with CaGPI2 expression levels. (i) and (ii) The hyphal growth and quantification of hyphal growth in various strains for up to 120 minutes in liquid RPMI with 10% serum media at 37 °C. A minimum of 100 cells were used for the statistical analysis. The experiment was repeated twice in duplicates; averages with standard deviations are shown. (B) CaERG11 transcript levels correlate with levels of CaGPI19 expression. The transcript levels of CaERG11 were monitored in the mutants strains relative to either BWP17 or BWP17URA3. (C) The azole response of the strains correlates with CaERG11 expression levels. (i) Strains overexpressing CaGPI19 were grown in liquid SD medium containing 0.2 µg/ml ketoconazole. (ii) Depletion of CaGPI19 slows down the doubling time of the CaGPI15Hz/CaGPI19Hz mutant when cultured with 0.08 µg/ml ketoconazole. Two independent experiments in duplicates were done for confirmation. (D) Rtt109 regulates CaERG11 transcription and sensitivity to azoles in the CaGPI19 mutant. (i) ChIP analysis was done to compare the H3K56Ac of CaERG11 promoter in the mutant strains relative to BWP17URA3. (ii) Transcript levels of RTT109 and CaERG11 were monitored in the mutant strains relative to BWP17URA3. (iii) Response to azoles was monitored in CaGPI19Hz/pACT1-RTT109 mutant. All strains were grown with or without 0.2 µg/ml ketoconazole and the doubling time was calculated (Supplementary Table 2). The experiments were repeated twice in duplicates. (iv) Relative quantification of ergosterol was done in different mutants of CaGPI19 using GC-MS. P-values for all heterozygous mutants were calculated with respect to the wild type controls while that for overexpression mutants are with respect to CaGPI19Hz-URA3.

three other downstream GPI biosynthetic genes (CaGPI12, CaGPI14, CaGPI8) were all found to not be significantly altered in the CaGPI15Hz or the CaGPI15 revertant strains (Supplementary Fig. 3A).

Further, the acetylation of histone H3 on the promoter of CaGPI15, CaGPI2 and CaGPI19 in the CaGPI15Hz strain was found to be significantly reduced when probed using anti-H3K56 antibody (Fig. 3B(i,ii)). Overexpression of RTT109 in CaGPI15Hz restored the H3Ac (Fig. 3B(ii)) and the levels of all three genes (Fig. 3B(iii)). It also reversed azole sensitivity as described above (Fig. 2E; Supplementary Fig. 4C), and filamentation of the CaGPI15Hz mutant (Fig. 3C). In the latter, where one allele of CaGPI15Hz was disrupted and in the latter strains were generated. In the former, one allele of CaGPI15Hz was disrupted in CaGPI15Hz background, filamentation was further reduced (Fig. 4A(i,ii)); Supplementary Fig. 3C). In the latter, where CaGPI2 was overexpressed in the CaGPI15Hz background, filamentation was restored (Fig. 4A(iii)); Supplementary Fig. 3C). Similarly, to confirm that CaGPI19 functions below CaGPI15 in controlling hyphal morphogenesis, we recently demonstrated that CaGPI2 regulates downstream CaGPI15 in controlling hyphal morphogenesis. Filamentation of two strains, CaGPI15Hz/CaGPI2Hz and CaGPI15Hz/pACT1-CaGPI2, confirmed this. In the former, where one allele of CaGPI15Hz is disrupted in CaGPI15Hz background, filamentation was further reduced (Fig. 4A(i,ii)); Supplementary Fig. 3C). In the latter, where CaGPI2 was overexpressed in the CaGPI15Hz background, filamentation was restored (Fig. 4A(iii)); Supplementary Fig. 3C). Similarly, to confirm that CaGPI19 functions below CaGPI15 in controlling CaERG11 levels and sensitivity toazole drugs, CaGPI15Hz/CaGPI19Hz and CaGPI15Hz/pACT1-CaGPI19 strains were generated. In the former, one allele of CaGPI19 was disrupted and in the latter CaGPI19 was overexpressed in the CaGPI15Hz background. CaERG11 transcripts were reduced in CaGPI15Hz/CaGPI19Hz and upregulated in CaGPI15Hz/pACT1-CaGPI19 as compared to the parent strain (Fig. 4B). The sensitivities of these strains to azoles also correlated with their CaERG11 levels. CaGPI15Hz/CaGPI19Hz was more sensitive to azoles while CaGPI15Hz/pACT1-CaGPI19 was resistant to azoles as compared to CaGPI15Hz (Fig. 4C(i,ii)); Supplementary Fig. 5B). Thus, CaGPI19 functions downstream of CaGPI15 in controlling sensitivity toazole drugs.

ChIP analysis showed that acetylation of H3 was reduced at the promoter of CaERG11 in CaGPI19Hz strain and was restored by overexpression of RTT109 in CaGPI19Hz background (Fig. 4D(i)). Overexpression of RTT109 in CaGPI19Hz background also caused an enhancement in CaERG11 transcripts (Fig. 4D(ii)), reversal of azole sensitivity (Fig. 4D(iii)); Supplementary Fig. 5C and restoration in ergosterol levels (Fig. 4D(iv)).
The cross-talk between CaGPI2, CaGPI15 and CaGPI19. The data presented in Fig. 3A, suggested that CaGPI15 is an activator of both CaGPI2 and CaGPI19. To examine the interaction between CaGPI2 and CaGPI19 in the CaGPI15 background, we studied two double heterozygous strains, CaGPI15Hz/CaGPI2Hz and CaGPI15Hz/CaGPI19Hz. The levels of CaGPI19 were increased in CaGPI15Hz/CaGPI2Hz while that of CaGPI2 were increased in CaGPI15Hz/CaGPI19Hz. Overexpressing either CaGPI2 or CaGPI19 in CaGPI15Hz resulted in downregulation of the other (Fig. 5A). Thus, the mutually negative regulation between CaGPI2 and CaGPI19 continues to function in the CaGPI15Hz strain.

CaGPI15 transcripts are upregulated in CaGPI2Hz and CaGPI19Hz (Fig. 5B). We also examined two strains that had simultaneous downregulation of CaGPI2 and CaGPI19. Transcripts of CaGPI15 decreased in both CaGPI15Hz/CaGPI2Hz and CaGPI15Hz/CaGPI19Hz strains (Fig. 5B). Overexpression of CaGPI15 in the wild type strain led to simultaneous upregulation of CaGPI2 and CaGPI19 (Fig. 5C). Similarly, overexpression of either CaGPI2 or CaGPI19 caused overexpression of CaGPI15 while maintaining the negative regulation between CaGPI2 and CaGPI19 (Fig. 5C). Thus, both CaGPI2 and CaGPI19 can independently activate CaGPI15 (Fig. 5D). CaGPI15 acts upstream and serves to activate both CaGPI2 and CaGPI19 (Fig. 5D).

GPI-GnT activity assays also corroborate such a model. Overexpression of CaGPI15 in CaGPI2Hz or CaGPI19Hz pushes up transcript levels of both CaGPI2 and CaGPI19 and causes enhanced GPI-GnT activity.
in comparison to the parent strains (Fig. 5E). However, due to the negative regulation between CaGPI2 and CaGPI19, overexpressing either CaGPI2 or CaGPI19 in the CaGPI15Hz strain cannot enhance its GPI-GnT activity (Fig. 5E).

**CaGPI15 heterozygous strain is more susceptible to killing by MH-S macrophage cells and is less able to kill the macrophage cell line.** We tested the effect of CaGPI15 knock-down on C. albicans virulence. For this, a murine alveolar macrophage cell line (MH-S) was co-cultured with BWP17 and CaGPI15Hz strain for 3 h (Fig. 6A,B) and 18 h (Fig. 6C,D). Both strains of Candida formed hyphae when co-cultured with MH-S cells for 18 h. BWP17 co-cultured with MH-S had 55% longer hyphae than CaGPI15Hz co-cultured with MH-S for 18 h (Fig. 6E). The internalization of C. albicans cells by MH-S was roughly similar for the CaGPI15Hz and BWP17 strains (Fig. 6F). This was also the case when phagocytosis was inhibited with the help of cytochalasin D (Cyt D) (Fig. 6F). Thus, mutation of CaGPI15 does not appear to alter the phagocytosis of C. albicans cells by either cytoskeleton-dependent or independent pathways. The colony forming units (CFU) recovered after incubation with the macrophage cells was significantly lower in CaGPI15Hz as compared to BWP17 (Fig. 6G).

Hence, more numbers of CaGPI15Hz cells were killed by MH-S in comparison to BWP17. In addition, more live MH-S cells were recovered after co-culture with CaGPI15Hz as compared to BWP17, suggesting that CaGPI15Hz was less virulent (Fig. 6H). There was also no difference in cell death by pyroptosis after co-culturing MH-S with BWP17 and CaGPI15Hz at 1:5 multiplicity of infection (MOI) for 3 h and 18 h (Fig. 6I). But there is a significant difference in pyroptosis between 3 h and 18 h for MH-S co-cultured with either strain. Similarly, there was no difference in cell death by apoptosis (Annexin V staining) when MH-S cells were co-cultured with either with...
BWP17 or CaGPI15Hz strain at 1:5 MOI for 18 h. Nevertheless, the level of apoptosis was significant in MH-S on infection with either strain of C. albicans for 18 h. So, C. albicans seems to cause MH-S macrophage cell death by apoptosis as well as pyroptosis.

### CaGPI15 heterozygous strain is more susceptible to killing by LA-4 epithelial cells and is less able to kill the epithelial cell line.

We further investigated the interaction of CaGPI15Hz cells with an epithelial cell line. Murine epithelial cell line LA-4 cells were co-cultured with BWP17 and CaGPI15Hz strain for 3 h (Fig. 7A,B) and 18 h (Fig. 7C,D). Both strains formed hyphae on co-culture with LA-4 cells for 18 h (Fig. 7C,D) but BWP17 exhibited 51% longer hyphae than CaGPI15Hz (Fig. 7E). CaGPI15Hz cells were more susceptible to killing by LA-4 cells as significantly lower CFU were obtained for CaGPI15Hz co-cultured with LA-4 in comparison to that for BWP17 (Fig. 7F). Phagocytosis of BWP17 cells by LA-4 cells was significantly higher than that of CaGPI15Hz cells at MOI 1:5 (Fig. 7G), suggesting a role for fungal GPI anchored proteins in their uptake.

However, the live cell recovery of LA-4 cells was significantly higher for those co-cultured with CaGPI15Hz versus those with BWP17 at MOI 1:5 (Fig. 7H). No significant difference in phagocytosis or live cell recovery was observed in experiments using either of these strains at MOI 1:1. Significantly lower pyroptosis (~30% of wild-type) was seen in LA-4 cells when co-cultured with 18 h with CaGPI15Hz in comparison to those co-cultured with BWP17. No pyroptosis was seen when LA-4 cells were infected with either strain for 3 h. Further, we found a small but significantly higher degree of apoptosis in LA-4 cells infected with BWP17 cells versus CaGPI15Hz after...
We discovered that simultaneous downregulation of CaGPI2 and CaGPI19 levels, respectively, in these strains. Further, the hyphal morphogenesis and azole drug response phenotypes correlated with changes in CaGPI15 and continued to be mutually negatively regulated. CaGPI15 is not observed in other organisms. In a series of papers establishing such a link in C. cerevisiae, the GPI-GnT complex interacts with and regulates two other important pathways, ergosterol biosynthesis and apoptosis and pyroptosis. The data from epithelial cell infection studies also supports the notion that the GPI anchor glycolipid is produced in the endoplasmic reticulum in 10–12 sequential biochemical steps. In lower eukaryotes this pathway is essential to the growth and viability of the organism while in higher eukaryotes it is critical only at certain stages of organismal development, such as in embryogenesis. Given their essentiality for eukaryotic pathogens, several steps of the pathway have been the focus of study as probable drug targets. However, isolating and studying the individual enzymes of the GPI biosynthetic pathway is challenging because it involves mostly multi-subunit membrane-bound enzymes of relatively low abundance. No high-resolution X-ray crystallographic data are available for any of the enzymes till date and there are no commercially available substrates for most steps of the pathway. The study of the GPI biosynthetic pathway in C. albicans is made more challenging due to its codon bias. C. albicans proteins heterologously expressed in another host may at times need to be codon optimised for function. Further, until recently no cell free assay system was available for the GPI biosynthetic pathway in this organism since protocols used to generate microsomes from the closely related yeast, S. cerevisiae, did not yield active microsomes from C. albicans.

Despite the many challenges, studying the GPI anchor biosynthesis of different organisms without depending solely on model organisms can be very rewarding. For example, the first and committed step of the GPI biosynthesis pathway in eukaryotes is an important site for regulation. The presence of poorly conserved accessory subunits to assist the highly conserved catalytic subunit suggests that the regulation of the GPI biosynthetic pathway could be mediated via these proteins. Some evidence in support of such a hypothesis also exists. In S. cerevisiae, Ras2 was shown to inhibit the GPI-GnT complex and vice versa while no such regulation was observed in mammals. Similarly, Dpm2 is known to regulate the mammalian GPI-GnT, but an equivalent regulation is not observed in other organisms. In a series of papers establishing such a link in C. albicans, we showed that the GPI-GnT complex interacts with and regulates two other important pathways, ergosterol biosynthesis and hyphal morphogenesis in C. albicans. Specifically, CaGpi19 controls CaERG11 levels and modulates azole drug response while CaGpi2 regulates hyphal morphogenesis by controlling Ras signaling. Moreover, CaRas1, the C. albicans homolog of S. cerevisiae Ras2, activates rather than inhibits the GPI-GnT activity. In turn, the CaRas1-dependent PKA signaling pathway is activated by CaGpi2, but this is independent of the GPI-GnT activity itself.

In the present study, we examined a third subunit of the GPI-GnT complex to elucidate its importance for C. albicans. CaGpi15, like CaGpi2 and CaGpi19, is a poorly conserved protein sharing very low sequence homology with its yeast counterpart. Yet, at the functional level it complements a conditionally lethal S. cerevisiae gpi15 mutant. Thus, significant functional similarities exist between the Gpi15 homologs in the two organisms. In C. albicans, as in S. cerevisiae, CaGpi15 appears to be important for cell growth. Gene-dosage effects also operate here since the heterozygous mutants were only marginally affected in doubling times while the conditional null showed significantly longer doubling times as compared to the wild type strains. Cell wall defects and clumping observed in CaGpi15 mutants appear to be a result of GPI anchor deficiency since these have also been observed in other GPI biosynthetic mutants.

CaGpi15 mutants were ergosterol deficient and sensitive to azoles. Thus, in its response to azoles, it appeared that CaGpi15 mutants mirrored the CaGpi19 mutants. Yet, surprisingly, the hyphal morphogenesis phenotype of CaGpi15 mutant cells was quite unlike the hyperfilamentous phenotype of CaGpi19 mutants. CaGpi15 mutants were hypofilamentous as compared to the wild type strains due to reduced Ras signaling, a feature also observed in CaGpi2 mutants. Thus, CaGpi15 exhibited phenotypes of a mutant that had both CaGpi2 and CaGpi19 downregulated. A transcript level analysis confirmed this. Significantly reduced histone H3 acetylation was observed on the promoters of CaGpi2, CaGpi15 and CaGpi19 in the CaGpi15 mutant strain. Overexpression of RTT109 could restore H3Ac as well as the transcript levels of the three genes, suggesting that expression levels of these genes are regulated via histone H3 acetylation in the CaGpi15 mutant strain. Overexpressing CaGpi15 could also restore CaGpi2 and CaGpi19 levels. Phenotypic assays as well as GPI-GnT activity assays corroborated these results.

Using double mutants in which one allele of either CaGpi2 or CaGpi19 was disrupted in the heterozygous CaGpi15 background, we discovered that CaGpi2 and CaGpi19 continued to be mutually negatively regulated. Further, the hyphal morphogenesis and azole drug response phenotypes correlated with changes in CaGpi2 and CaGpi19 levels, respectively, in these strains.

Finally, we addressed the question of whether CaGpi2 and CaGpi19 were activators or repressors of CaGpi15. We discovered that simultaneous downregulation of CaGpi2 and CaGpi19 in C. albicans results in downregulation of CaGpi15 and overexpression of either of them activates CaGpi15. In other words, both CaGpi2 and CaGpi19 could independently activate CaGpi15. Thus, we propose a model for how the three subunits of the GPI-GnT complex interact in C. albicans (Fig. 5D): CaGpi15 stimulates activation of CaGpi2 as well as CaGpi19. Downregulating it can simultaneously decrease levels of both CaGpi2 and CaGpi19. In turn, both CaGpi2 and CaGpi19 can independently activate CaGpi15. Disrupting either CaGpi2 or CaGpi19 results in upregulation of the other, which in turn upregulates CaGpi15. Both CaGpi2 and CaGpi19 are mutually negatively regulated and function downstream of CaGpi15 as far as hyphal growth and azole drug response are concerned. Sensitivity to azoles via CaERG11 regulation correlates with CaGpi19 levels while Ras-dependent hyphal morphogenesis correlates with CaGpi2 levels.

Two additional issues needed to be addressed here. The first is the mechanism by which CaERG11 is downregulated in the CaGpi15 mutant. The presence of Upc2, a transcription factor for CaERG11, was found to be reduced on the promoter of the CaERG11 gene in CaGpi15 deficient strain. In exploring the possible reason...
for this, it was observed that H3Ac on the promoter of CaERG11 is also reduced in the azole sensitive CaGPI15 mutant strain. Since CaGPI19 functions downstream of CaGPI15, this strain was also tested for H3Ac and found to have reduced levels of it on the CaERG11 promoter. This is specifically due to loss of Rtt109 activity in these strains. Overexpressing RTT109 could restore the levels of the GPI-GnT subunits as well as of CaERG11 and reverse the response to azoles for both CaGPI15 and CaGPI19 mutant strains.

The second is the mechanism by which Ras signaling is altered in the CaGPI15 mutant. Overexpressing CaRAS1, but not CaRAS2, could restore filamentation in CaGPI15 mutant. Further, CaGPI2 functions downstream of CaGPI15 in this. In a recent manuscript, we showed that CaGpi2 physically interacts with CaRas1 in the endoplasmic reticulum and this interaction helps CaRas1 activate GPI-GnT activity37. CaGpi2 also regulates Ras signaling that occurs at the plasma membrane to trigger hyphal morphogenesis, but it does so by its effect on Hsp90 levels in the cell. It is well known that Hsp90 along with its co-chaperone, Sgl1, interacts with Cyt1, the effector of CaRas1, thereby preventing the interaction of GTP-bound CaRas1 with Cyt1 for initiating cAMP-dependent hyphal morphogenesis38. Overexpressing CaGPI2 results in downregulation of Hsp90 and a lifting of the inhibition exerted by Hsp90 on the Ras signaling pathway. Since CaGpi2 seems to control hyphal morphogenesis in the CaGPI15 mutant strain, it is reasonable to expect that this mechanism functions here too.

The CaGPI13 heterozygous mutant was also far more susceptible to killing by macrophages and epithelial cells in our assays. It showed reduced internalization by epithelial cells, and also shorter hyphal length in comparison to BWP17. In addition, its ability to damage macrophages and epithelial cells was significantly lesser than that of the wild type strain. The damage caused to macrophages and epithelial cells by infection with BWP17 and CaGPI15 heterozygous mutant involved apoptosis as well as pyroptosis, but significantly the pyroptosis seen in epithelial cells on infection with CaGPI15 heterozygous mutant was only 30% of that seen on infection with BWP17. This could be an important contributing factor in reduced damage to epithelial cells by this mutant. Hence, we infer that the CaGPI15 mutant strain is attenuated in virulence.

In conclusion, what is evident from our previous and current studies is that alterations in levels of different subunits of the same complex, all of which affect GPI biosynthesis, receive varying responses from ergosterol biosynthesis and hyphal filamentation pathways in C. albicans. Given the importance of GPI biosynthesis for the viability and growth of C. albicans, the multiple modes of interaction and regulation probably allow the GPI biosynthetic pathway to rapidly respond to multiple signals.

The clinical implications of these observations are hard to miss. Hyphal filamentation and invasive growth are important for the establishment of infection by C. albicans and several hyphae-specific factors are known to be GPI anchored34. Strains with reduced levels of GPI anchored proteins are known to show attenuated virulence. Additionally, ergosterol and its biosynthetic pathway continue to be the most important targets of the currently available antifungals. However, one of the major problems in treatment of fungal infections has been the rapid drug resistance that develops upon continued usage of these drugs33. CaGPI15 as an alternative target, a master regulator that simultaneously affects hyphal morphogenesis as well as ergosterol biosynthesis could be an interesting candidate. Indeed, strains in which CaGPI15 levels are reduced are less virulent, as shown here. Additionally, GPI biosynthesis itself is an essential pathway in C. albicans and targeting the first step of GPI biosynthesis via a protein that bears little homology with its mammalian counterpart could be an effective strategy.

Methods

Strains and media. Yeast and C. albicans strains used in this study are described in Table 1. Murine alveolar epithelial type I cell line (LA-4) and Murine alveolar macrophage cell line (MH-S) were procured from the American type tissue culture collection (ATCC), Rockville, MD, USA. All primers used in this study are listed in Supplementary Table 1. Strains were grown in yeast extract-peptone-dextrose (YEPD) media or synthetic dextrose minimal media (SD media). Ura− strains were grown in YEPD or SD media supplemented with 60 µg/ml uridine. Similarly, His− strains or Arg− strains were grown in SD medium supplemented with the appropriate amino acid (85.6 µg/ml His or Arg). Transformations were performed using lithium-acetate method34. RPMI-1640 medium was from Sigma, MO, USA and FBS from Gibco, Life technologies, Grand Island, NY, USA. RNA PolII (sc-21750) from Santa Cruz Biotechnology, Histone H3 (mAb-96C10) from Cell Signaling, H3K56Ac (ab71956) from Abcam and H3K9Ac (mAb-H0913) from Sigma.

Chemicals. Chemicals were purchased from Merck, Qualigens, or Sigma-Aldrich (USA) unless specified otherwise. Enzymes were from Bangalore Genei (India), MBF Fermentas (USA) or New England Biolabs (USA). Gel extraction and PCR purification kits were from Qiagen. Primers were synthesized by GCC Biotech (India).

PCR amplification of CaGPI15 gene. The putative CaGPI15 gene sequence was obtained from Prof. Eisenhaber’s web site (http://mendel.imp.ac.at/SEQUENCES/gpi-biosynthesis/pigs-main.html) and also confirmed by BLASTp analysis using the mammalian PIG-H sequence as query. Forward primer FPCaGPI15-pCaDis and reverse primer RPCaGPI15-pCaDis (Supplementary Table 1) were used to amplify CaGPI15 gene from genomic DNA of C. albicans. The amplified product was visualized on 1% agarose gel.

Chromosomal disruption of CaGPI15 to make heterozygous CaGPI15 mutant. CaGPI15 heterozygote in BWP17 strain of C. albicans (CaGPI15Hz) was made by PCR-mediated disruption using HIS1 as a selection marker35,37.

Construction of regulatable null mutant of CaGPI15 gene. Conditional null mutant of CaGPI15 in BWP17 strain (Cagpi15 null) was made by placing the functional gene in CaGPI15Hz under the MET3 promoter using PCR mediated approach36.
grown for 16 h in 4% (w/v) galactose. It was inoculated into 250 ml SD medium containing either 4% galactose or 2% glucose at 30 °C till OD₆₀₀nm of 2.0 was reached. The cells were harvested and microsomes prepared from it as described previously.

Preparation of microsomes from yeast and GPI-GnT assay.  Primary cultures of yeast strains were grown for 16 h in 4% (w/v) galactose. It was inoculated into 250 ml SD medium containing either 4% galactose or 2% glucose at 30 °C till OD₆₀₀nm of 2.0 was reached. The cells were harvested and microsomes prepared from it as described previously.

GPI-GnT activity of the microsomes was assayed as described previously. The assay produces both [6³H] GlcNAc-Pi (N-acetyl glucosaminylphosphatidylinositol) and [6³H]GlcN-Pi (glucosaminylphosphatidylinositol, the product of the next step of the pathway) and were detected using Bioscan AR2000 TLC scanner. The sum of the radioactive counts detected for the two products in the case of the control strain was considered to be 100%
and GPI-GnT activity of all strains was calculated relative to it. In all cases averages of data from independent experiments done twice in duplicates along with standard deviations are plotted.

Preparation of microsomes from *C. albicans* and GPI-GnT assay. Microsomes from *C. albicans* were prepared using a protocol previously standardized in our lab with minor modifications. GPI-GnT assay was performed using microsomes corresponding to ~1500µg protein as described previously. Comparable amount of heat-killed microsomes were used as the negative control for the assay.

Generating the CaGPI15 revertant strain. *CaGPI15* revertant strain was previously reported. The pACT1-CaGPI15 construct was used to transform *CaGPI15Hz* cells and the positive clones were confirmed by PCR. The control strains, BWP17/URA3 and *CaGPI15Hz-URA3* were previously reported.

Construction of other overexpression strains in the heterozygote mutants. *CaGPI15Hz/pACT1-CaGPI2* strain was previously reported. Using the same strategy, plasmid, pACT1-CaGPI19 was used to generate *CaGPI15Hz/pACT1-CaGPI19*. Plasmid pACT1-CaRAS1 or pACT1-CaRAS2 was used to generate *CaGPI15Hz/pACT1-CaRAS1* and *CaGPI15Hz/pACT1-CaRAS2*, respectively. The plasmid pACT1-RTT109 was used to generate *CaGPI15Hz/pACT1-RTT109* and *CaGPI19Hz/pACT1-RTT109* strains. BWP17/pACT1-CaGPI2 strain was previously reported. The overexpression strains BWP17/pACT1-CaGPI19 and BWP17/pACT1-CaGPI15 were similarly made. The control strain *CaGPI19Hz-URA3* was previously reported. Positive colonies were confirmed by PCR using appropriate primers (Supplementary Table 1).

Generation of double heterozygous strains. *CaGPI15Hz/CaGPI19Hz* and *CaGPI15Hz/CaGPI2Hz* were generated using the strategy previously described. Confirmation was done by using gene-flanking primers (Table 1).

Generation of VPS75Hz strain. *CaVPS75Hz* in BWP17/URA3 strain of *C. albicans* (CaVPS75Hz-URA3) was made by PCR-mediated disruption using ARG4 as a selection marker. Confirmation was done by using gene-flanking primers (Table 1).

Monitoring growth rate in liquid cultures. Growth rate of different mutants was monitored by plotting growth curves. Primary cultures were grown overnight in liquid SD medium at 30 °C, 220 rpm. Secondary cultures were set to an initial OD600nm of 0.2 in 60 ml fresh medium and subjected to shaking at 220 rpm, 30 °C. OD600nm was measured at every 2 h interval to assess the growth in each strain till the saturation was reached. Doubling times were calculated by plotting the exponential growth phase of the cells between 4 and 10 h (OD600nm vs time) for each strain. All the experiments were done twice in duplicates.

Hyphal induction. Hyphal growth was monitored in liquid and solid media using the method described previously. All experiments were done at least thrice in duplicates using independent cultures.

Azole response of mutants. Sensitivity of the strains to azole drugs was studied by spot assays as well as by growth in liquid medium in the presence of ketoconazole. Primary and secondary cultures were set as described earlier in 'Monitoring growth rate in liquid cultures' section. Final concentration of ketoconazole (0.08 or 0.2 µg/ml) was maintained in 60 ml of 0.2 OD600nm secondary cultures. The way of collecting the samples for growth measurement and calculating the doubling times were same as described earlier in this section. Two independent experiments with duplicates were done for each set of the mutants.

Estimation of sterol levels. The levels of ergosterol and lanosterol were quantified by GC-MS as described previously. Data in all graphs are averages of two independent experiments done in duplicates along with standard deviations.

Quantification of transcript levels through RT-PCR. RNA extraction, cDNA preparation and quantification of transcript levels was done as described previously using RT primers (Supplementary Table 1). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was taken as an internal control. Data in all graphs are averages with standard deviations of two experiments done in duplicates using independent cultures.

Cell Clumping Assay. Cells were grown in minimal media at 30 °C, 220 rpm until saturation. Cells from 500µl culture were pelleted down at 5000 rpm, 5 min, washed in 1X PBS and resuspended in 50% glycerol. A 5µl cell suspension was spotted on microscopic slides and observed under Nikon Eclipse Ti Microscope. Cells were quantified for studying cell aggregation.

Calcoflour white (CFW) staining and Congo red (CR) staining in the cell wall. Sample preparation was done as described previously. Cells were stained with 100µg/ml CFW or 100µg/ml CR for 30 min at 30 °C, washed thrice with PBS and observed under a Nikon SMZ TiE fluorescence microscope. Fluorescence intensity was quantified using NIS Elements AR Version 4 software.

Heat sensitivity assays. A 10 min heat shock exposure at 48 °C was given and plate assays done as at 30 °C for 48 h described earlier.
Chromatin Immunoprecipitation (ChiP) assay. ChIP was carried out as described previously. Briefly, cells were harvested after cross-linking DNA to protein with 1% (v/v) formaldehyde. Glycine (25 mM) was added and cells lysed by glass beads in lysis buffer (50 mM HEPES, pH 7.4; 140 mM NaCl; 1 mM EDTA; 1% Triton X-100; 1 mM PMSF). After sonication the supernatant was incubated overnight with 1 µg anti-RNA PolII or H3K56Ac antibody. Protein-A-CL agarose beads (20 µl) were added. The beads were spun down after 2 h, washed with lysis buffer, high-salt buffer (lysis buffer with 500 mM NaCl), wash buffer (10 mM Tris-Cl, pH 8.0; 250 mM LiCl; 1 mM EDTA; 0.5% NP-40) and Tris-EDTA buffer. Eluates collected in elution buffer (150 µl Tris-EDTA buffer containing 1.0% SDS) were treated with 20 µg proteinase K. DNA was separated using phenol:chloroform (1:1) and precipitated. The samples were analyzed by PCR using primers for specific regions of promoters.

Cell line and Maintenance of cell line. MH-S and LA-4 cell line were cultured and maintained in RPMI 1640 media, supplement with 25 mM HEPES (Sigma, MO, USA) (N-[2-[100 hydroxyethyl] piperazine-N-[2-ethanesulfonic acid]), 50 µg/ml gentamicin sulfate, 0.05 mM 2-mercaptoethanol, 300 µg/ml L-glutamine and 50 µg/ml and 10% heat inactivate FBS (Gibco, Life technologies, Grand Island, NY, USA) in a humidified atmosphere containing 5% CO₂ at 37 °C. Cell lines were maintained as adherent cultures and sub cultured by trypsinization. The cells were then harvested by using 2.5% w/v trypsin from bovine pancreas in 10 mM EDTA disodium salt to detach the monolayer. The harvested cells were collected and pelleted down by centrifugation at 240 g for 5 min at 4 °C. The pellet of cells were suspended in 1 ml media and counted for viability by haemocytometer using trypan blue dye.

Uptake of *C. albicans*. CFSE labelling of *C. albicans*. *C. albicans* cells (100 million) were labelled with 10 µM carboxyfluorescein N-succinimidyl ester (CFSE) as described previously. MH-S or LA-4 cells (0.3 million) cultured in 24-well plate were incubated with CFSE labelled BWP17 and CaGPI15Hz at different MOI such as 1:1 and 1:5 for 3 h and 18 h at 37 °C in CO₂ incubator. The cells were trypsinized and then harvested with PBS. The harvested cells were fixed in 2% PFA and the uptake of stained BWP17 and CaGPI15Hz by MH-S or LA-4 cells was assessed by using BD FACS Calibur flowcytometer in FL1 channel using Cell Quest software.

Co-culture assay in vitro. Co-culture assay was done as described previously. MH-S cells or LA-4 cells (0.3 million) were incubated with CFSE labelled fungal cells for 3 h at 1:1 or 1:5 MOI. The phagocytosis-independent uptake of fungal cells by MH-S was monitored using 2.5 µg/ml of Cyt D, an inhibitor of actin polymerization as described previously.

Confocal microscopy. For visualization of uptake of *C. albicans* by MH-S or LA-4 cells, 0.3 million cells were cultured on glass cover slips overnight. Cells were then co-cultured with CFSE-labelled BWP17 and CaGPI15Hz at MOI (1:5) for 3 h and 18 h at 37 °C. Cells were then washed, fixed with 2% paraformaldehyde (PFA) followed by washing thrice with quencher (ammonium chloride) and examined using a confocal laser scanning microscope (Olympus FluoView FV1000). Five images each were captured having Z sections (depths 0.1 µm). The above co-cultured cells of 18 h time point were used to determine the length of hyphae. Nikon NIS element software was used to measure the length of the hyphae.

Macrophage or epithelial cell mediated killing of BWP17 and CaGPI15Hz. The macrophage or epithelial cell mediated killing of *C. albicans* was studied as described previously. The number of *C. albicans* was determined as CFU/ml = number of colonies x dilution factor / volume of culture plate.

Detection of pyroptosis through lactate dehydrogenase (LDH) enzymatic assay. The macrophage or epithelial cell mediated killing of *C. albicans* was studied as described previously. The number of *C. albicans* was determined as CFU/ml = number of colonies x dilution factor / volume of culture plate.

Assessment of apoptosis. LA-4 and MH-S cells were cultured at a concentration of 0.3 million cells/ml in a 24 well cell culture plates. After overnight culture, cells were washed with complete medium to remove debris and dead cells. Cells were co-cultured with BWP17 and CaGPI15Hz for 3 h and 18 h at different MOI (1:5) at 37 °C in CO₂ incubator. The co-cultured cells were then subjected for cell cytotoxicity assay. The cell cytotoxicity assay was performed using Cytotoxicity Detection Kit (Pierce™, USA). The standard protocol assay reported here were performed according to the manufacturer's instructions. The amount of LDH released either in control (Positive control, spontaneous and maximum release) or in the experimental wells was used to calculate the % specific lysate. The % specific lysis was calculated as: % Specific lysis = (Experimental release – Spontaneous release) × 100/(Maximum release – Spontaneous release).

Assessment of apoptosis. LA-4 and MH-S cells were cultured at a concentration of 0.3 million cells/ml in a 24 well cell culture plates. After overnight culture, cells were washed with complete medium to remove debris and dead cells. Cells were co-cultured with BWP17 and CaGPI15Hz for 18 h at MOI (1:5) at 37 °C in CO₂ incubator. The cells were then washed with PBS to remove BWP17 and CaGPI15Hz. The cells were trypsinized and washed followed by staining with annexin V APC conjugate (Biolegend, San Diego, CA, USA) to assess the apoptotic cells using BD FACS Calibur™ and analyzed through Cell Quest Software.

Statistical significance of data. Unless otherwise stated, statistical significance of the data (p value) was calculated in Sigma plot 8.0 and GraphPad Prism 5.0 using Student’s t-test. The p value > 0.05 is considered not significant and is depicted by n.s., p value ≤ 0.05 is depicted by *p value ≤ 0.005 is depicted by **p value ≤ 0.0005 is depicted by ***.
Data Availability
The datasets and material generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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**Acknowledgements**

SSK received funding from Department of Biotechnology (DBT) India (BT/PR3749/BRB/10/986/2011), Department of Science and Technology (DST) India (SB/SO/BB-011/2014) as well as from an umbrella grant to the School of Life Sciences from the Department of Science and Technology under Promotion of University Research and Scientific Excellence (DST-PURSE No. SR/PURSE/PHASE 2/11 (C)). PJ, PG, SCS, SLS, BY, PK, UY, NN and R were supported by Senior and Junior Research fellowships from Council of Scientific and Industrial Research or University Grants Commission or Indian Council of Medical Research, India. Two of the strains, *CaGPI15Hz* and *Cagpi15 null* were generated by Kalpana Pawar while being employed in a funded research project of SSK. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. We thank Dr. Aaron P. Mitchell for the BWP17 strain, Dr. Marwan Al-Shawi for the YEpHIS vector, Dr. W.A. Fonzi for pMB7 vector, Prof. P.E. Sudbery for pCaDis vector, Dr. Cheryl Gale for URA3-pMET3-GFP vector and Prof. Alistair JP Brown for pACT1-GFP vector. We thank Dr. Ajai Kumar for the GC-MS runs. The GC-MS data was recorded at the Advanced Instrumentation Research Facility, JNU. Fluorescence microscopy images were recorded at the Central Instrumentation Facility, SLS, JNU.

**Author Contributions**

PJ, P.G., S.C.S., N.N., B.Y., P.K., S.L.S., U.Y., S.B., R. executed the experiments. S.S.K., R.M., P.J. and P.G. planned the experiments and data analysis. S.S.K., P.J., P.G. wrote the manuscript with inputs from R.M. and N.P. All authors gave final approval for publication.

**Additional Information**

Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-019-44919-4.

**Competing Interests:** The authors declare no competing interests.

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