Crystal structure of \textit{Gib2}, a signal-transducing protein scaffold associated with ribosomes in \textit{Cryptococcus neoformans}

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The atypical Gβ-like/RACK1 \textit{Gib2} protein promotes cAMP signalling that plays a central role in regulating the virulence of \textit{Cryptococcus neoformans}. \textit{Gib2} contains a seven-bladed \textit{β}-transducin structure and is emerging as a scaffold protein interconnecting signalling pathways through interactions with various protein partners. Here, we present the crystal structure of \textit{Gib2} at a 2.2-Å resolution. The structure allows us to analyse the association between \textit{Gib2} and the ribosome, as well as to identify the \textit{Gib2} amino acid residues involved in ribosome binding. Our studies not only suggest that \textit{Gib2} has a role in protein translation but also present \textit{Gib2} as a physical link at the crossroads of various regulatory pathways important for the growth and virulence of \textit{C. neoformans}.

\textit{Cryptococcus neoformans}, an encapsulated yeast-like basidiomycetous fungus, is the primary culprit behind fungal meningoencephalitis in immune-compromised individuals¹. \textit{C. neoformans} affects approximately one million people worldwide, primarily in developing countries, and accounts for over 600,000 fatalities annually²–⁴. The relevance and genetic tractability has enabled \textit{C. neoformans} to emerge as a model organism to study the molecular mechanisms of fungal pathogenesis.

Factors important for the virulence of \textit{C. neoformans}, such as the antioxidant melanin pigment and anti-phagocytic capsule, are regulated by the conserved cAMP-dependent signalling pathway mediated by the GTP-binding (G) protein \textit{γ} subunit Gpa1⁴–⁶. Gpb1, the only known classical G protein \textit{β} subunit in \textit{C. neoformans}, regulates pheromone-responsive mating and haploid differentiation through association with the G protein \textit{γ} subunits Gpa2 or Gpa3 and \textit{γ} subunits Gpg1 or Gpg2, but has not been implicated in Gpa1-cAMP signalling⁷–¹². Although a classical G protein \textit{β} subunit was not found for Gpa1 in \textit{C. neoformans}, Palmer and co-workers reported that a Gpa1-interacting protein, \textit{Gib2}, could function as an atypical G protein \textit{β} subunit¹³. \textit{Gib2} binds directly to Gpa1 and likely facilitates its oscillation between the active and inactive states, thereby affecting cAMP signalling¹³. \textit{Gib2} was also shown to promote cAMP levels in cells lacking Gpa1 presumably by relieving the inhibitory effect of Ras1 protein on adenylyl cyclase Cac1 protein in the absence of Gpa1¹³,¹⁴. In addition to Gpa1, \textit{Gib2} physically interacts with Gpg1 and Gpg2, as well as with a downstream target of Gpa1-cAMP signalling, Smg1 protein, and several other proteins¹³,¹⁴.

Similar to Gpb1, \textit{Gib2} contains a seven Trp-Asp (WD) repeat motif¹⁵. The WD repeat family of proteins comprise polypeptide stretches of 40–60 residues that each fold into a four-stranded antiparallel \textit{β}-sheet. Hence, \textit{Gib2} was likewise predicted to fold into a seven-bladed \textit{β}-propeller, similar to that seen in the crystal structure of \textit{β} transducin¹⁵,¹⁶. Indeed, modelling revealed that the \textit{Gib2} and Gpb1 structures were similar¹⁵. However, based on the amino acid sequence analysis, \textit{Gib2} is more closely related to RACK1 (receptor for activated kinase C) protein orthologues than to G protein \textit{β} subunits¹³,¹⁴. For example, \textit{Gib2} shares 70% amino acid sequence identity with mammalian RACK1 but only 25% with \textit{C. neoformans} Gpb1¹⁵. The high sequence similarity shared between \textit{C. neoformans} \textit{Gib2} and the extensively studied human RACK1, as well as Asc1 protein (the RACK1 orthologue in...
Saccharomyces cerevisiae) (Fig. 1) suggests that Gib2 could have functions similar to those of the aforementioned proteins.

Human RACK1 orthologues are scaffold proteins that integrate numerous cellular processes (e.g., development, neuropathology, and cellular stress) through interacting with as many as 80 estimated protein partners, among which are kinases (e.g., PKC, Src, and FAK), phosphatases, membrane receptors (e.g., integrin β subunits), and G proteins17–21. RACK1 presumably recruits these proteins to their appropriate subcellular sites, thereby integrating various intracellular signalling pathways 22. The deletion of RACK1 orthologues is lethal in higher eukaryotes, whereas the consequence of Asc1 deletion is less severe in S. cerevisiae23.

As mentioned above, Gib2 directly interacts with the G proteins Gpa1, G pg1, and G pg213,14. Gib2 also physically interacts with the protein kinase C homologue Pkc113. Using GST affinity purification combined with mass spectrometry, Wang and co-workers identified approximately 50 proteins that interact with Gib2, including proteins involved in signalling, intracellular trafficking, stress responses, and metabolism14. Interestingly, a significant proportion of the identified proteins are involved in protein translation and ribosome composition14. The same finding was also seen in RACK1 and Asc1 interactomes14,24,25. In fact, both RACK1 and Asc1 have been shown to be the ribosomal core proteins associated with the 40S ribosomal subunit25–28.

Hence, Gib2, a scaffold protein interconnecting various cellular processes through binding a myriad of proteins, could also bind with the ribosome and function in ribosomal biogenesis and protein translation. By recruiting various proteins to ribosomes, Gib2 may act as a link between protein translation and other cellular processes. To better illustrate such functions, we here present the crystal structure of Gib2 and show its interaction with ribosomes of C. neoformans. We also present predictions of Gib2 residues involved in the association with ribosomes and discuss the role that Gib2-ribosome binding may play in the virulence of C. neoformans.

Results

Effect of GIB2 disruption on C. neoformans growth. The C. neoformans species includes two highly relevant but distinct varieties, var. grubii and var. neoformans. Gib2 was originally reported to have an essential function in C. neoformans var. neoformans (serotype D)
because the knockdown of GIB2 by antisense suppression resulted in a severe growth defect, and no GIB2 deletion strains linked to the auxotrophic marker Ura5 could be recovered. However, the GIB2 deletion strains could be readily recovered if dominant selective marker genes were used. The GIB2 deletion strains displayed no reduction in the cAMP levels or apparent defects in melanin and capsule formation, suggesting that they are not directly linked to virulence. However, based on spotting a serially diluted cell culture on medium plates, the growth of the GIB2 deletion strain was reduced at 37°C but not at 30°C or 23°C. In addition, mice infected with the GIB2 deletion strain survived nearly twice as long as those infected with the wild-type strain. Apparently, although not essential, Gib2 is important for growth at the mammalian body temperature and is required for full virulence.

To accurately assess the effect of GIB2 disruption on the viability of C. neoformans, growth curves of the two deletion strains and their parental strain H99 were determined at 30 °C and 37 °C in rich YPD and nutrient-limiting YNB media. Although the two strains exhibited similar growth profiles at 30°C, the GIB2 deletion strain showed approximately a two-fold reduction in growth in YPD media at 37°C (Fig. 2, left panel). This finding is in agreement with that of previous studies by Wang and co-workers. The effect of GIB2 deletion on C. neoformans growth is even more pronounced in YNB medium and can be observed even at 30°C (Fig. 2, right panel). It was reported previously that a higher level of Gib2 expression could be found when the cells were switched to YNB medium. Thus, Gib2 is responsive to nutrient deprivation conditions.

Ribosome binding of Gib2. Basic cellular functions, such as ribosomal biogenesis and protein translation, underlie the growth and differentiation of eukaryotic cells. Mammalian RACK1 and S. cerevisiae Asc1 proteins, to which Gib2 shares high homology, are core ribosomal proteins that regulate growth in response to stresses, such as elevated temperature. To test whether C. neoformans Gib2 can form a complex with ribosomes as a basis of the thermal response, we assessed the binding of a recombinant (His-tagged) Gib2 to 80S ribosomes purified from wild-type H99 and the GIB2 deletion strain. For comparison, the binding of human RACK1 to C. neoformans ribosomes was also tested. Following incubation with either Gib2 or RACK1, ribosomes were precipitated through a sucrose cushion by centrifugation, and the associated proteins were separated using SDS-PAGE. As a control, Gib2 and RACK1 proteins were loaded onto sucrose cushion in the absence of ribosomes. For reference, purified Gib2 and RACK1 proteins were directly loaded onto SDS-gel as well. The recombinant Gib2 and RACK1 were visualised by Western blotting using the anti His-tag and anti-RACK1 antibodies, respectively.

Our results revealed that the recombinant Gib2 binds to ribosomes from both wild-type (WT) and the GIB2 deletion (Agib2) strain (Fig. 3A). However, binding to the GIB2 deletion strain ribosomes is more efficient, indicating that endogenous Gib2 protein co-purifies with ribosomes from the wild-type strain. Our data suggest that, similar to RACK1 and Asc1 proteins discussed above, Gib2 is a core component co-purifying with the ribosomes. When the endogenous Gib2 is not present in C. neoformans cells, the recombinant Gib2 can bind to ribosomes in vitro. That recombinant Gib2 can bind to ribosomes isolated from the wild-type strain, albeit less efficiently, indicates that a proportion of native Gib2 is exchanged in the binding assay, although it is possible that isolated wild-type ribosomes are not “saturated” with the native Gib2. The human RACK1 was able to bind to C. neoformans ribosomes (Fig. 3B), further highlighting the conservation between Gib2 and RACK1.

Crystal structure determination. We determined the crystal structure of Gib2 at 2.2-Å resolution by molecular replacement using Asc1 (PDB ID: 3FRX) as a search model. The asymmetric unit contained one copy of Gib2. Data collection and refinement statistics are given in Table 1. The model (Fig. 4) includes all of the C. neoformans Gib2 residues (314 in total), except for Met1. The electron-density map is well defined apart from the first and last residue, as well as a short stretch of side chains between residues Gly276 and Arg282 in the extended loop linking blades six and seven (Fig. 1 and Fig. 4). The structure features the predicted seven β-propeller fold, with an overall shape that resembles a donut (Fig. 4) of approximate dimensions 45 Å and 10 Å at the outer and inner circle, respectively, and 30 Å in width. When observed from the side, one rim is slightly narrower, resulting in a conical overall appearance. Moreover, all of the seven β-propeller blades are arranged radially around the central axis and comprise four twisted antiparallel β-sheets labelled A, B, C, and D (Fig. 1 and Fig. 4), starting from the inside. Neighbouring blades are connected by loops linking D and A sheets, along with loops connecting inter-blade B and C sheets, which are exposed at the narrower rim. The loops connecting inter-blade A and B, as well as C and D sheets, are exposed at the larger rim interface of the propeller (Fig. 1 and Fig. 4).

The interactions stabilising the β-propeller fold are conserved in blades one to five. The aromatic side chains of the conserved Trp residues of the WD repeat (Fig. 1) point to the hydrophobic space between the blades and make interactions with Ser (Thr in blade one) ten residues downstream through hydrogen bond (Fig. 5). The Ser residue also makes hydrogen bond interactions with His residues from the conserved GH motifs in the loops connecting neighbouring blades, which in turn contacts with conserved Asp residues downstream of the Trp through hydrogen bonds (Fig. 5). The network of inter-blade hydrogen bonds between conserved residues observed in blades one to five is absent in blades six and seven. In blade six, Phe residue replaces the WD motif Trp residue. Although the corresponding Trp residue is present in blade seven (Fig. 1), its orientation differs from the one observed in blades one to five. Deviation from the conserved structural motif in blades six and seven might be necessary to accommodate the extended loop located between these two blades (Fig. 4) or possibly provide a more dynamic binding site for protein partners.

Gib2 structure comparisons. The structure of C. neoformans Gib2 showed good agreement with those of human RACK1 (PDB ID 4AOW) and S. cerevisiae Asc1 (PDB ID 3FRX) with Gib2 being more similar to RACK1 than to Asc1 because it contains one less residue and is less flexible in Asc1 (Fig. 6). Additionally, the inner (A) β-sheets are slightly more centrally oriented in Gib2 (Fig. 6B). As the extended loop between blades six and seven displays higher sequence variability among Gib2, RACK1, and Asc1 compared to that in other regions (Fig. 1), it likely exhibits a higher degree of flexibility in protein structures. In accordance, this region has a less well-defined electron density map for side chains of various residues in both Gib2 and Asc1 (Fig. 6A) and was not visible in the electron-density map of RACK1. Nonetheless, the electron density for the backbone of the extended loop was sufficient to shed light on the overall conformation of the loop region in Gib2 (Fig. 6C). Although the middle portion of the loop seems to have a different conformation in Gib2 and Asc1, the knob-like structure in Asc1 consisting of stacked Pro276, Phe278, and Pro287 is also observed in the Gib2 structure (consisting of Pro272, Phe274, and Pro284, respectively) (Fig. 6D). This knob-like structure is most likely not present in RACK1, as the corresponding residues are Gly272, Val274, and Pro284, respectively (Fig. 1). The loop region is likely less flexible in Asc1 because it contains one less residue and is further stabilised by an edge-to-face π-π interaction involving Tyr281. The latter is replaced by Leu and Thr in Gib2 and RACK1 proteins, respectively (Fig. 1).
Although Gib2 crystallises as a monomer, Western blotting has revealed that it can form dimeric complexes under physiological conditions. The structural characterisation of yeast ribosomes showed that Asc1 co-purifies with ribosomes in the monomeric form. However, the crystal structure of the Asc1 homodimer revealed that, at least in solution, oligomerisation could occur through the reorganisation of blades four of both monomers, creating a shared blade and exposing a different surface to potential binding partners. A small fraction of human RACK1 protein is also present in the oligomeric form, mostly as dimers in solution. The proportion of dimeric RACK1 seems to depend on conditions, such as salt concentration, pH, and temperature. Deletion analyses revealed that homodimerisation involves blade four in mammalian RACK1 as well. Whether Gib2 oligomerises in a manner similar to Asc1 and RACK1, as well as the functional significance of oligomerisation, remain to be determined.

**Model for Gib2 bound to the ribosome.** Cryo-EM studies of *S. cerevisiae* ribosomes indicated that Asc1 is located in the head region of the 40S subunit close to the mRNA exit tunnel. Further comparison with other available cryo-EM and crystal structures of eukaryotic ribosomes confirmed that the location and orientation of RACK1 orthologues on ribosomes are conserved in eukaryotes. We superimposed the Gib2 structure onto Asc1 in the presence of the crystal structure of *S. cerevisiae* ribosome (PDB: 3U5B and 3U5C) and found that the interactions are likely to occur between Gib2 blades one and two, the negatively charged phosphate backbone of ribosomal small subunit RNA helices 39 and 40, and ribosomal proteins rpS16e and rpS17e (Fig. 7). An interaction between Gib2 blade five and the C-terminal tail of rpS3e is also likely, as it can be observed in the crystal structure of *Tetrahymena thermophila* 40S ribosome. It should be stressed, however, that the structural similarity of *C. neoformans* ribosomes to other eukaryotic ribosomes is not known.

**Gib2 interacts with eIF4A.** The D-E-A-D (Asp-Glu-Ala-Asp)-box containing RNA helicases are essential ribosomal components serving as initiation factors in protein translation in eukaryotic cells. An interaction between Gib2 and the *C. neoformans* eIF4A (eukaryotic translation initiation factor 4A) homologue was established previously by Wang et al. To elaborate the association between Gib2 and the ribosome, we assayed the interaction between...
Gib2 and eIF4A through the co-immunoprecipitation of heterologously expressed proteins. Consistent with the previous study 14, eIF4A (expressed in pET-32a, instead of pRSET-B) was pulled down by Gib2 (expressed in pGEX-6P, instead of pET41a(1)) (Fig. 3C).

Discussion

C. neoformans is a fungal pathogen that causes life-threatening infections primarily in individuals with compromised immune systems. The virulence of C. neoformans is regulated by the cAMP-dependent signalling pathway4–8. Although Gib2 has been reported to have a role in cAMP signalling by promoting cAMP levels in cells lacking G protein α subunit Gpa1, a key factor in cAMP-dependent regulation of virulence3,13,14, disruption of the GIB2 gene in C. neoformans serotype A, affected neither cAMP levels nor pigment and capsule formation14. Nonetheless, murine virulence assays revealed that Gib2 regulates virulence characteristics indirectly. Our study validates the previous findings by others and further elevates the study by presenting the crystal structure of Gib2, a key regulatory protein in C. neoformans.

Based on its homology to mammalian RACK1 and yeast Asc1 (Fig. 1), well-known scaffold proteins linking several signalling pathways17,22, Gib2 has been predicted and shown to have multiple functions in C. neoformans13,14. We propose that Gib2 is structurally similar to RACK1 and Asc1 and that Gib2 is associated with ribosomes as well. Indeed, we were able to determine the crystal structure of C. neoformans Gib2 at a 2.2-A˚ resolution. The Gib2 structure features the β-propeller fold with each of the seven blades consisting of four antiparallel β-sheets (Fig. 4) that show overall good agreement with both Asc1 and RACK1 structures (Fig. 6). In addition, we showed that both the C. neoformans Gib2 and the human RACK1

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can form a complex with *C. neoformans* ribosomes *in vitro* (Fig. 3). Furthermore, based on the crystal structure of yeast ribosome in complex with Asc1, we modelled the interaction between Gib2 and ribosome (Fig. 7). In *S. cerevisiae* Asc1, there are several conserved, positively charged, and solvent accessible residues that serve as the main association sites for ribosomal binding, e.g., the conserved Arg38-Asp39-Lys40 within the first WD-40 domain are of significance, underlined by the finding that this region is responsible for the decrease in tolerance to translation inhibitors. Intriguingly, these residues are also present in Gib2 (Fig. 7B). Asc1 Arg38 (Arg36 in Gib2) and Lys40 (Lys38) contribute to ribosome binding both in *in vitro* and in *in vivo* binding assays. Mutations of Lys62, Lys87, Arg90, and Arg102 (correspond to His60, His85, Arg88, and Arg100 in Gib2, respectively) caused defects in Asc1-ribosome association. Asc1 Arg38 (Arg36 in Gib2) and Lys40 (Lys38) contribute to ribosome binding both in *in vitro* and in *in vivo* binding assays. Mutations of Lys62, Lys87, Arg90, and Arg102 (correspond to His60, His85, Arg88, and Arg100 in Gib2, respectively) caused defects in Asc1-ribosome association. Asc1 Arg38 (Arg36 in Gib2) and Lys40 (Lys38) contribute to ribosome binding both in *in vitro* and in *in vivo* binding assays. Mutations of Lys62, Lys87, Arg90, and Arg102 (correspond to His60, His85, Arg88, and Arg100 in Gib2, respectively) caused defects in Asc1-ribosome association. Asc1 Arg38 (Arg36 in Gib2) and Lys40 (Lys38) contribute to ribosome binding both in *in vitro* and in *in vivo* binding assays. Mutations of Lys62, Lys87, Arg90, and Arg102 (correspond to His60, His85, Arg88, and Arg100 in Gib2, respectively) caused defects in Asc1-ribosome association.

**Table 1 | Data collection and refinement statistics**

| Data collection |
|-----------------|
| **Space group** | P41 | 21 |
| **Cell dimensions** | a, b, c (Å) | 81.7, 81.7, 136.0 |
| **Rsym (%)** | 50.0–2.2 (2.35–2.2)* |
| **I/σI** | 13.5 (2.5) |
| **CC1/2 (%)** | 99.8 (91.8) |
| **Completeness (%)** | 99.9 (99.9) |
| **Redundancy** | 7.7 (5.9) |

| Refinement |
|------------|
| **Resolution (Å)** | 50.0–2.2 |
| **No. of reflections** | 187,760 |
| **Rwork/Rfree (%)** | 19.5/23.1 |
| **B-factors** | Protein 52.1 |
| **R.m.s. deviations** | Bond lengths (Å) 0.009 |
| **Bond angles (°)** | 1.3 |

*Values in parentheses are for the highest-resolution shell.*

**Figure 4 | The crystal structure of Gib2.** Cartoon (A and C) and surface (B and D) representation of the *C. neoformans* Gib2 crystal structure viewed from the top (A and B) and the side (C and D). Molecules are coloured using the chainbow scheme from blue (N-terminus) to red (C-terminus) and visualised using MacPyMOL software. The seven β-propeller blades are numbered, and individual β-sheets for the second blade are also labelled (A). The extended loop between blades six and seven is also indicated (B).
orthologues (Fig. 1). Based on sequence (Fig. 1) and structural (Fig. 6) similarity to mammalian RACK1 and yeast Asc1 discussed above, we propose that Gib2’s positively charged and surface accessible residues Arg36, Lys38, Lys57, His60, His85, Arg88, and Arg100 contribute to the interactions with rRNA (Fig. 7).

The orientation of previously studied RACK1 orthologues and, therefore, highly likely Gib2 on the ribosome, suggests that, while binding per se could stabilise the 40S subunit, it should not significantly affect ribosome functioning in translation. However, the larger rim face and the sides of blades four to seven of ribosome-bound RACK1 orthologues are solvent accessible and, hence, good candidates for creating binding sites for several protein interaction partners. Moreover, the side of blades five and six of ribosome-bound RACK orthologues face the mRNA exit tunnel. This implicates a potentially significant functional consequence of Gib2-ribosome binding.

There are several examples of protein binding to the four to seven blade region of RACK1/Asc1 proteins. For instance, protein kinase C (PKC) can bind to blade six as revealed by peptide mapping studies. In addition, a recent affinity grid-based cryo-EM study of PKC binding to RACK1 on the ribosome suggests that it binds to the blade three and four region as well. Although a physical interaction between Pkc1 (PKC homologue in C. neoformans) and Gib2 has been reported, the region of Gib2 involved in complex formation needs further identification. Additionally, Src kinase binds to and phosphorylates conserved Tyr residues located at the edge of blades five and six in RACK1. However, no Src kinase homolo-

Figure 5 | Conserved interactions in Gib2 blades one to five. Blade two is shown with individual β-sheets labelled. The side chains of conserved residues are shown in sticks, and the tertiary interactions stabilising the blade are shown in the dashed line.

Figure 6 | Gib2 comparison with RACK1 and Asc1. Superimposition of C. neoformans Gib2 in green, human RACK1 (PDB ID 4AOW) in purple, and S. cerevisiae Asc1 (PDB ID 3FRX) in light blue. A, B, and C viewed from the top, bottom, and side, respectively. Close up of the extended loop region (D) is also shown. Side chains of the Asc1 residues involved in the knob-like structure are shown as sticks and labelled individually.
Gibbs have been identified in *C. neoformans*. Other findings, such as the effect of phosphorylation of RACK1 on its interactions with β-integrins and Kindlin-3 involving blades five to seven, cannot be validated because of the lack of comparable homologue proteins in *C. neoformans*. However, eukaryotic translation initiation factor 3 (eIF3) was reported to associate with ribosomes through binding to the one to three blade region of Asc1. It is conceivable that such a binding pattern would also apply to Gib2 because the interaction between Gib2 and eIF4A was validated under two different testing conditions (Fig. 3 and Wang et al. 14). Modelling of the Asc1 homodimer on the 40S subunit also revealed the feasibility of multimeric complex formation, suggesting that Gib2 could employ similar oligomerisation strategies to regulate the recruitment of binding partners to the ribosome.

Examples of the influence of ribosome-bound RACK1 on translation in different eukaryotes are accumulating. For instance, RACK1 stimulates translation by recruiting activated PKC to 40S subunits, and the PKC dependent phosphorylation of eIF6 on 60S subunits leads to subunit association. Accordingly, heterozygous RACK1 gene depletion in mice caused the accumulation of monosomes and impaired protein synthesis. RACK1 also recruits the stress induced c-Jun N-terminal kinase (JNK) to ribosomes, where it phosphorylates the eukaryotic translation elongation factor 1A isoform 2 (eEF1A2) and promotes the degradation of newly synthesised poly-peptides (NSP), thereby establishing a role for RACK1 in the quality control of NSPs under stress conditions. In *S. cerevisiae*, the deletion of the ASC1 gene affected the phosphorylation of eukaryotic translation initiation factors 2 (eIF2) and 4A (eIF4A), affinity of eIF3 and eIF5 binding to 40S ribosomes, and assembly of the pre-initiation complex. These findings indicate an important regulatory role of Asc1 in general translation initiation, and provide compelling reasons for the presence of similar functions by Gib2.

In addition to affecting general translation, RACK1 and Asc1 were shown to regulate the translation of specific mRNAs. For instance, RACK1/Asc1 regulates the translation initiation of specific mRNAs through their respective 5' UTR sequences, which could be...
mediated by interactions between RACK1 and ZBP1, or Asc1 and Sc160p\(^{1,2}\). RACK1/Asc1 could therefore mediate the delivery of specific mRNAs close to the mRNA binding site on 40S ribosome. Based on the high conservation, it is conceivable that Gib2 may also exhibit similar functions.

An interactome analysis showed that Gib2 interacts with more than 50 proteins\(^{3,4}\). In addition to numerous proteins involved in signalling (e.g., Pkc1, Cac1, Rsa1), response to chemical stimuli (e.g., Gpa1), transport, and various other cellular processes, a significant proportion of Gib2 binding partners are ribosome related, either ribosomal core components (e.g., RPS3, RPS7, RPL4, RPL6, RPL13, and RPL19) or translation factors (e.g., eEF1A, eEF4)\(^{3,4}\). Therefore, we propose that the structure of Gib2 provides a platform for multiple binding partners and allows the ribosome-bound Gib2 to function as a hub linking diverse cellular processes (e.g., signalling, stress response, intracellular trafficking) to translation in C. neoformans.

Thus, Gib2 has both ribosome-independent and -dependent functions in C. neoformans. Gib2 interacts with G protein subunit Gpa1 and assists its functions in the cAMP-signalling pathway to regulate virulence (melanin pigment and capsule formation) of C. neoformans\(^{3,4}\). We suggest that ribosome-bound Gib2 may regulate translation by responding to environmental changes (e.g., higher temperature upon infecting mammals) through interacting with proteins involved in various signalling pathways. Gib2 could affect the functioning of translation factors leading to changes in translation efficiency, the recruitment of specific mRNA to ribosomes, or intracellular trafficking of ribosomes. In the absence of Gib2, C. neoformans may face challenges in adjusting to changes in the living environment, leading to reduced fitness and virulence.

Finally, Gib2 is emerging as a link between diverse cellular processes, virulence, and translation regulation in the widely spread and precarious human pathogen C. neoformans. Solving the crystal structure of Gib2 sheds light onto its versatile functions as a ribosome-bound scaffold for numerous binding partners. It also provides a basis for future studies, such as the mutagenesis of Gib2, binding assays to identify/confirm its binding partners, the structural characterisation of these protein complexes to reveal the virulence mechanism, and drug target identification for antifungal therapy.

**Methods**

**Strains, media, and growth conditions.** C. neoformans wild-type H99 and Gib2 deletion strain in 5 L of YPD (2% glucose, 1% yeast extract, 2% yeast extract, 2% yeast extract, 2% yeast extract) and stored at -80°C.

Ribosome isolation. Three to five grams of fungal cells were re-suspended in 2 ml of buffer A (50 mM Tris-HCl pH 7.0, 50 mM NaCl, 10 mM magnesium acetate, 100 mM EDTA, 5 mM DTT, 0.2 mM PMSF, and 10% glycerol) and transferred into 1 ml aliquots in 2 ml cryotubes containing 0.5 mg of glass beads (460 μm). Cells were lysed using a Precellys 24 (Bertin Technologies, France) tissue homogeniser (6400 rpm, three times for 60 sec).

The crude extract was precipitated by centrifugation at 3000 rpm for 1 min. The lysate was centrifuged at 5000 rpm for 10 min, and the supernatant was centrifuged twice at 10,000 rpm for 15 min. The supernatant was then centrifuged at 50,000 rpm for 3 hours. The pellet was re-suspended in buffer B (buffer A with 500 mM KCl) and centrifuged at 5000 rpm for 10 min. The supernatant was overlaid on a 25% glycerol cushion in buffer C and centrifuged at 24,000 rpm for 3 hours. The pellet was re-suspended in buffer A with 10% sucrose and centrifuged at 5000 rpm for 10 min. The supernatant was diluted two-fold with buffer A (without sucrose or glycerol), layered onto 10 to 30% sucrose gradient in buffer A and centrifuged at 19,000 rpm for 17.5 hours using SW 28 Ti type rotor (Beckman Coulter Inc. US).

The ribosome profile was determined by continuous monitoring of A260, 80S ribosome-containing fractions were pooled. Ribosomes were precipitated by centrifugation at 40,000 rpm for 20 hours, re-suspended in buffer G (10 mM Hepes-KOH pH 7.5, 50 mM KOAc, 10 mM NH4Cl, 5 mM Mg(OAc)\(_2\), and 2 mM DTT), and stored at -80°C.

Gib2 expression and purification. BL21(DE3)pLysS competent cells were transformed with the pTOPHis-Lip-Gib2 plasmid and grown in 5 L of 2YT media (16 g of Bacto tryptone, 10 g of Bacto yeast extract, and 5 g of NaCl per L) supplemented with ampicillin and chloramphenicol. When the OD600 reached 0.3, the temperature was lowered to 16°C; when the OD600 reached 0.8-0.9, iso-prolly B-D-1-thiogalactopyranoside (IPTG, 2.5 mM) was added to induce Gib2 expression. Cells grown overnight were harvested by centrifugation and stored at -80°C. Cells were re-suspended in 50 ml Tris-HCl pH 8.0, 200 mM NaCl, and 5 mM β-mercaptoethanol and lysed using a Panda homogeniser (GE Naro Soavi, Italy). The lysate was centrifuged at 20,000 rpm for 20 min. The supernatant was the loaded onto a HisTrap HP Q-5 ml column (GE Healthcare, UK) equilibrated with the same buffer. Imidazole was used for protein elution. Gib2-containing fractions were pooled and concentrated to 10 ml before loading onto a HiLoad 26/60 Superdex 75 pg column (GE Healthcare). Column pre-equilibration and protein elution were performed using buffer containing 20 mM Tris-HCl pH 8.0, 50 mM NaCl, and 5 mM β-mercaptoethanol. Gib2 appeared as a single peak and the corresponding fractions were pooled, concentrated, and stored at -80°C.

**In vitro ribosome binding assay.** 80S ribosomes (1.5 μM) were incubated with 6 μM Gib2 (with His-tag, 46.2 kDa) or human RACK1 (~40 kDa) in buffer G in a final volume of 25 μl at 30°C for 25 min. The reaction mixture was filtered through a 0.22-μm filter, the volume was adjusted to 50 μl with buffer G, and layered onto 200 μl of 1.1 M sucrose cushion followed by centrifugation at 45,000 rpm for 18 hours at 4°C. For control, Gib2 and RACK1 proteins without 80S ribosomes were layered onto sucrose cushion in parallel. The pellet was washed once and dissolved in buffer G. Samples were analysed by SDS-PAGE and Western blotting analysis following standard protocols. The anti-His and anti-RACK1 antibodies were used for detecting Gib2 and RACK1, respectively.

Co-immunoprecipitation. Gib2 and eEF4A cDNAs were cloned into vectors pGEX4T-2 and pET32a, respectively, and transformed into Rosetta 2(DE3) cells (Novagen, US). 1 L of LB medium was inoculated with 5 ml of overnight culture and grown at 37°C until OD600 reached 0.5. IPTG (0.1 mM final concentration) was added to induce protein expression at 25°C. After 8 hours, cells were harvested by centrifugation and stored at -70°C. To extract proteins, cells were suspended in lysis buffer (20 mM Tris-HCl pH 7.4, 0.15 M NaCl, 0.5 mM EDTA, 1% Triton X-100, 1 mM DTT, and 1 mM PMSF) and lysed with sonication (2 sec pulses with 4 sec pauses for 2 min). Crude extracts were centrifuged (13,000 rpm for 15 min at 4°C) and supernatants were recovered. In case of GST-tagged Gib2, the supernatant was mixed with glutathione-sepharose resin (Amersham Pharmacia, US) for 2 h. The slurry mixture was then centrifuged at 500 rpm for 2 min at 4°C, washed with Tris-NaCl buffer (50 mM Tris-HCl pH 7.4, 100 mM NaCl, 1% Triton X-100, and 1 mM PMSF), and the protein was eluted by adding 15 mM glutathione. The eluted protein was dialysed overnight against Tris-NaCl buffer using Slide-A-Lyzer cassette (Thermo Fisher Scientific, US), recovered, and verified by SDS-PAGE and Western blotting analysis with the anti-GST antibody (M20007, Abmart, China). Preparation of the His-tagged eEF4A protein was similar to the above except that crude protein extract was mixed with His-Select Nickel affinity gel (Sigma-Aldrich, US), eluted with Tris-NaCl buffer containing 200 mM imidazole, and dialysed as above. Target protein was verified by Western blotting analysis with the anti-GST antibody (M20001, Abmart, China). For binding, 500 μl of GST–Gib2 protein was added to glutathione-sepharose resin in 1 ml of GST binding buffer. 500 μl of His–eEF4A protein was then added and the mixture was incubated overnight with gentle rotation at 4°C. The resin was precipitated and washed three times with Tris-NaCl buffer. 100 μl of Tris-NaCl buffer and 25 μl of 5X protein sample buffer were then added to the resin before denaturing by boiling for 5 min. 4 μl of the sample was then separated by SDS-PAGE, transferred to the PVDF membrane, and visualized using the anti-GST and anti-His antibodies. For the negative control, the GST protein was used as an input.

Crystallisation. The TEV protease-cleaved Gib2 (~9.3 mg/ml) was used for crystallisation trials. Initial crystal hits were found using commercial screens: Crystal Screen (Hampton Research, US) and syntax (Hampton Research, US). 10% glycerol was used in BTE (400 mM bicine, pH 9.5, 500 mM NaCl, and 30% PEG 3350) and 20% PEG 400 in buffer G. Crystals appeared in 96-well plates. Crystals of ~400 × 50 × 50 μm grew at 18°C when 0.2 ml of the well solution (100 mM sodium citrate (pH 6.5) and 1 M sodium citrate) was mixed with 0.2 μl protein sample. For cryo protection, 30% PEG3350 was added. Crystals were mounted and flash frozen in liquid nitrogen.

Data collection, processing, and model building. Diffraction data were collected on the PXI beamline at the Swiss Light Source (SLS) at a 1-A wavelength using a Pilatus 6 M detector (Dectris, Switzerland) at 100 K. The data were processed using X-ray
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**Author contributions**

Y.G. and R.E. designed the project and wrote the manuscript. R.E. prepared figures 1–6. Y.C. prepared figure 7. R.E., V.T.D., S.F. and W.B. purified the proteins and ribosomes, as well as carried out the experiments. T.L., P.W. and C.X. provided *C. neoformans* strains. C.X., P.W. and S.M.T. contributed to research discussion and manuscript finalisation. All of the authors reviewed the manuscript.

**Additional information**

**Accession codes:** The coordinates and structure factors for Gib2 have been deposited in the PDB with accession code 4D6V at 2.2-Å resolution.

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