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Metabolic modulation: *Pneumocystis* phosphoglucomutase is a target influencing host recognition

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ABSTRACT

Herein, this manuscript explores the significance of the phosphoglucomutase (PGM) enzyme in *Pneumocystis* spp., focusing on its role in fungal surface mannoprotein formation. Through expression of the *Pneumocystis murina Pmpgm2* in a *Saccharomyces cerevisiae pgm2Δ* strain, we demonstrate restoration of binding to the mannose receptor (MR) and macrophages to wildtype yeast levels in this complemented strain. Gas Chromatography-Mass Spectroscopy (GC-MS) confirmed reduced mannose content in the *pgm2Δ* yeast strain compared to the wild-type and complemented *Pmpgm2* cDNA-expressing strains. This study underscores fungal PGM function in dolichol glucosyl phosphate biosynthesis, crucial for proper cell wall mannoprotein formation. Furthermore, highlighting the conservation of targetable cysteine residues across fungal pathogens, PGM inhibition maybe a potential therapeutic strategy against a broad spectrum of fungal infections.

Pneumocystis is a fungus belonging to the phylum Ascomycota (Gigliotti et al., 2014). *Pneumocytis jirovecii* is one of the most common opportunistic pathogens in individuals with HIV infection (Huang et al., 2011). While the advent of highly active antiretroviral therapy has decreased the incidence of Pneumocystis jirovecii Pneumonia (PJP) in HIV (Lopez-Sanchez et al., 2015); the incidence of non-HIV-infected patients with PJP appears to be increasing, and the mortality rate remains unacceptably high (Pates et al., 2023; Wang et al., 2022). The *Pneumocystis* cell surface is decorated with β-1,3 and β-1,6 glucans along with the most abundant cell surface component of the organism, the major surface glycoprotein (MSG; also known as gpA) (Kottom et al., 2015; Kutty et al., 2013; Kutty et al., 2008; Kottom and Limper, 2000; De Stefano et al., 1998). MSG is a glycoprotein complex that is not highly mannosylated, with the M5N2 being the predominant N- linked glycan present on *Pneumocystis* MSG-proteins (Ma et al., 2016). MSG is expressed on both cyst and trophic forms of the organism, but more abundantly present on the trophic stage (Evans and Garvy, 2018). MSG appears to be involved in attachment of the organism to alveolar epithelial cells, as well as to macrophages via binding to C-type lectin receptors (CLRs) including the mannose receptor (MR) (Kottom et al., 2019; Sassi et al., 2018; Ezekowitz et al., 1991).

Recently, we reported the presence and biochemical characterization of the *Pneumocystis jirovecii* and *Pneumocystis murina* phosphoglucomutases (PGMs) in *Pneumocystis*. Both PGMs complemented the *Saccharomyces cerevisiae pgm2*Δ strain in similar fashion and also displayed similar phosphoglucomutase activity, indicating their substantial homology and cross function (Kottom et al., 2024). In yeast, PGMs are important for carbohydrate metabolism through the interconversion of glucose 1-phosphate (Glc-1-P) and glucose 6-phosphate (Glc-6-P), a vital step in UDP pools for β-glucan cell wall formation (Yan et al., 2022). Less appreciated and understudied is the potential role of PGM activity on the expression of fungal surface mannoproteins. In this study, we analyzed the ability of the *Pneumocystis murina Pmpgm2* cDNA expressed in the *Saccharomyces cerevisiae pgm2*Δ strain versus the yeast wild- type counterpart to bind a panel of hFc fusion C-type lectin receptor (CLR) carbohydrate recognition domains (CRDs), as well as the binding of these yeast constructs to the mouse macrophage RAW cell line. As Fig. 1 shows of the four Fc-fusion CRD proteins tested, the *pgm2*-null yeast was significantly altered in the ability to bind the mannose receptor (MR) CRD as compared to the wild-type and *Pmpgm2* restored counterparts (**A**). Furthermore, the deleted *pgm2* yeast were similarly defective in their ability to bind the RAW macrophage cell line (**B**). Analysis of the yeast cell wall composition by Gas Chromatography-Mass Spectroscopy (GC–MS) revealed that the *pgm2*Δ yeast strain contains 2-3X less

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mannose than the wild-type and complemented *Pmpgm2* cDNA-expressing counterparts (Fig. 2A, Table 1).

In addition to the importance of PGM in yeast during glucan synthesis from donor uridine diphosphate glucose, with the conversion of glucose-6-phosphate to glucose-1-phosphate by the enzyme (Yan et al., 2022), PGM also has an important role in the dolichol glucosyl phosphate biosynthetic pathway and in the synthesis of Dol-P-glucose (Burda and Aebi, 1999), which can serve as a substate for the glycosyltransferases Alg6p and Alg8p (Reiss et al., 1996). These glycosyltransferases are vital for proper cell wall mannan biosynthesis in *Saccharomyces cerevisiae* as well as in *Candida albicans* (Engering et al., 2002). Our data presented here further provide evidence for the importance of PGM activity in proper yeast cell wall mannoprotein formation. Although as mentioned above, the most abundant N- linked glycan configuration is the M5N2 configuration in *Pneumocystis*, the organism does contain minor amounts of longer mannan chains as long as M9N2 (Ma et al., 2016). Others have reported that *S. cerevisiae* contains highly complexed branch mannans (Engering et al., 2002; Giaimis et al., 1993), and we recently demonstrated that an MR Fc fusion could bind these yeast mannan to a significant degree (Kottom et al., 2019). Our results suggest that fungal PGMs are highly conserved and can function in the formation of both short as well as extended/complex N-linked glycan outer chain formation. Therefore, specifically targeting fungal PGMs might be an attractive therapeutic option against fungal pathogens. For example, recently, a specific inhibitor targeting the *Aspergillus fumigatus* PGM (*Af*PGM) termed ISFP10 with a IC50 value of 2 μM and 50-fold selectivity against the human PGM homolog has been reported (Yan et al., 2022) and also used by our lab to show significant reduction in PmPgm2 activity in vitro (Kottom et al., 2024). In *A. fumigatus*; this small molecule targets the cysteine at amino acid position 353 (C353) of the *Af*PGM, which is absent from the *Homo sapiens* PGM enzyme, accounting for the inhibitor's selectivity (Yan et al., 2022). Protein alignments of this region from ten of the top WHO fungal priority pathogen (Burki, 2023) PGM enzymes show conservation of this cysteine across 8 of the 10 fungal pathogens (Fig. 2B, red box), with only substitution of the amino acid from both of the *Cryptococcus* spp's. These results suggest targeting fungal PGM proteins with such specific inhibitors and thus affecting downstream synthesis of nascent β-glucan and N-linked mannoprotein formation might be a viable therapeutic strategy against a broad spectrum of fungal pathogens.

Two experiments and an analysis of the yeast cell wall composition were performed. All yeast experiments were conducted with yeast grown in 2 % galactose URA- minimal media to induce the pYES2.1/*Pmpgm2* or pYES2.1/*lacZ* (control) vectors, respectively (Kottom et al., 2024). First, to determine the percent binding of select hFc-fusion carbohydrate recognition binding domains (CRDs) to the respective yeast strain, 2.0 μg per well of native yeast protein lysate were deposited onto 96-well microtiter plates and left to incubate at 4 °C overnight. The next day, plates underwent three washes with 100 μl PBS-Tween (PBS-T). Subsequently, wells were blocked with PBS/10 % FBS/2.5 % milk at 4 °C for 2 h. After three additional washes with PBS-T, CLR hFc- fusion proteins (200 ng) (Kottom et al., 2019) in lectin binding buffer (LBB) (50 mM HEPES, 5 mM MgCl2, and 5 mM CaCl2) were incubated in the plate wells for 2 h at 4 °C. Following this, a 1:1000 dilution factor of HRP goat anti-human antibody (SouthernBiotech) in blocking buffer was applied for 1 h at 4 °C. Finally, after washing the plates three times with 1X PBS-T, 1X TMB substrate was added for 20 min at room temperature, followed by stopping the reaction with 2.0 M H2SO4. The plates were then analyzed using a VERSAmax microplate reader (Molecular Devices) at 450 nm. To analyze yeast construct binding to RAW macrophages, 2 x 10^5 cells were plated in duplicate in a 96 well microtiter plate and incubated for 1.5 h at 37 °C/5%C02. Next, yeast cells were added to the RAW cells at ~ 1:1 ratio. The plates were centrifuged at 500 x g to synchronize infection and allowed to incubate for 30 min. After 30 min, wells were washed 3 times with 1X PBS (200 ul/well), lysed with RIPA buffer incubation for 10 min and 50 ul plated onto 2 % galactose URA-

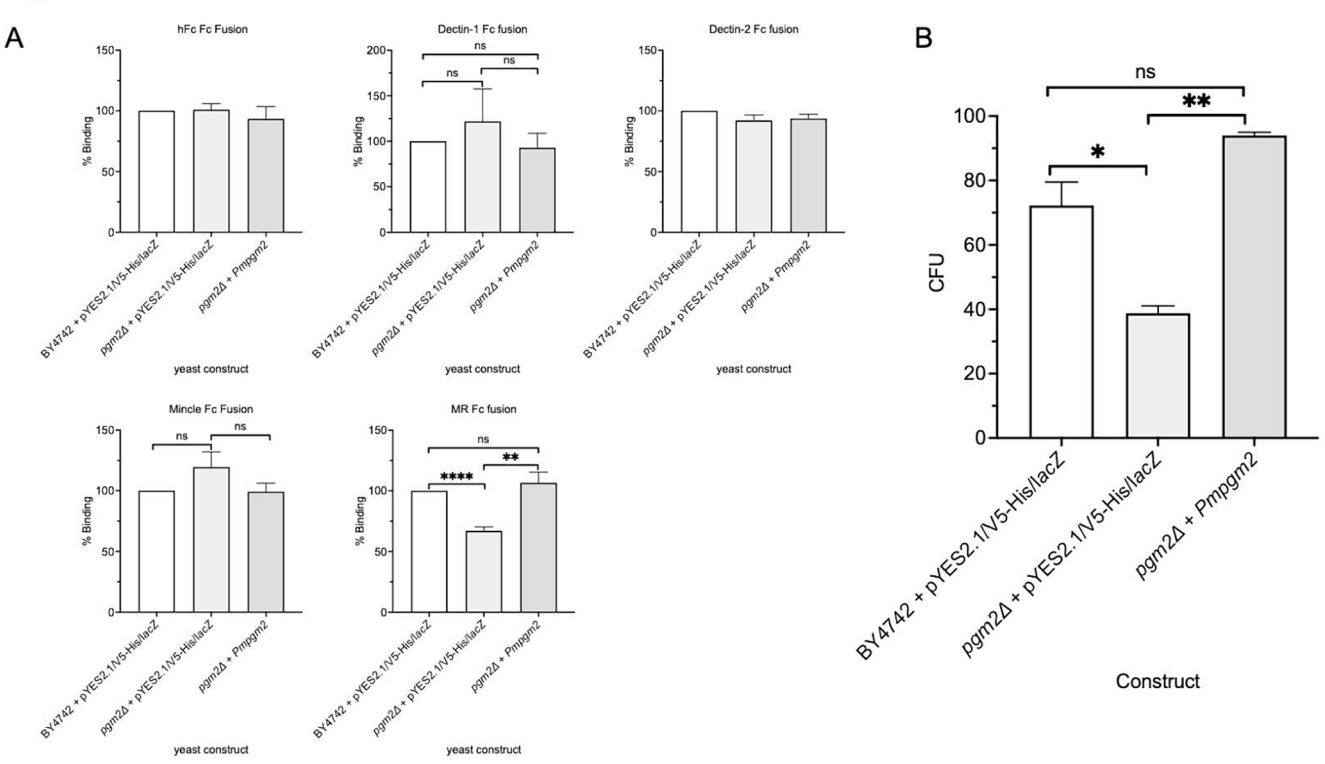

Fig. 1. **(A)** Percentage of respective yeast strain binding to CLR hFc-fusions. **(B)** Colony forming unit (CFU) counts of the respective bound/internalized yeast strains. Data analysis was initially first performed with ANOVA. If ANOVA indicated overall differences, subsequent group analysis was then performed by a two-sample unpaired Student *t* test for normally distributed variables. Error bars show SD from the mean. $^*P < 0.05$, $^{**}P < 0.01$, ns = non-significant.

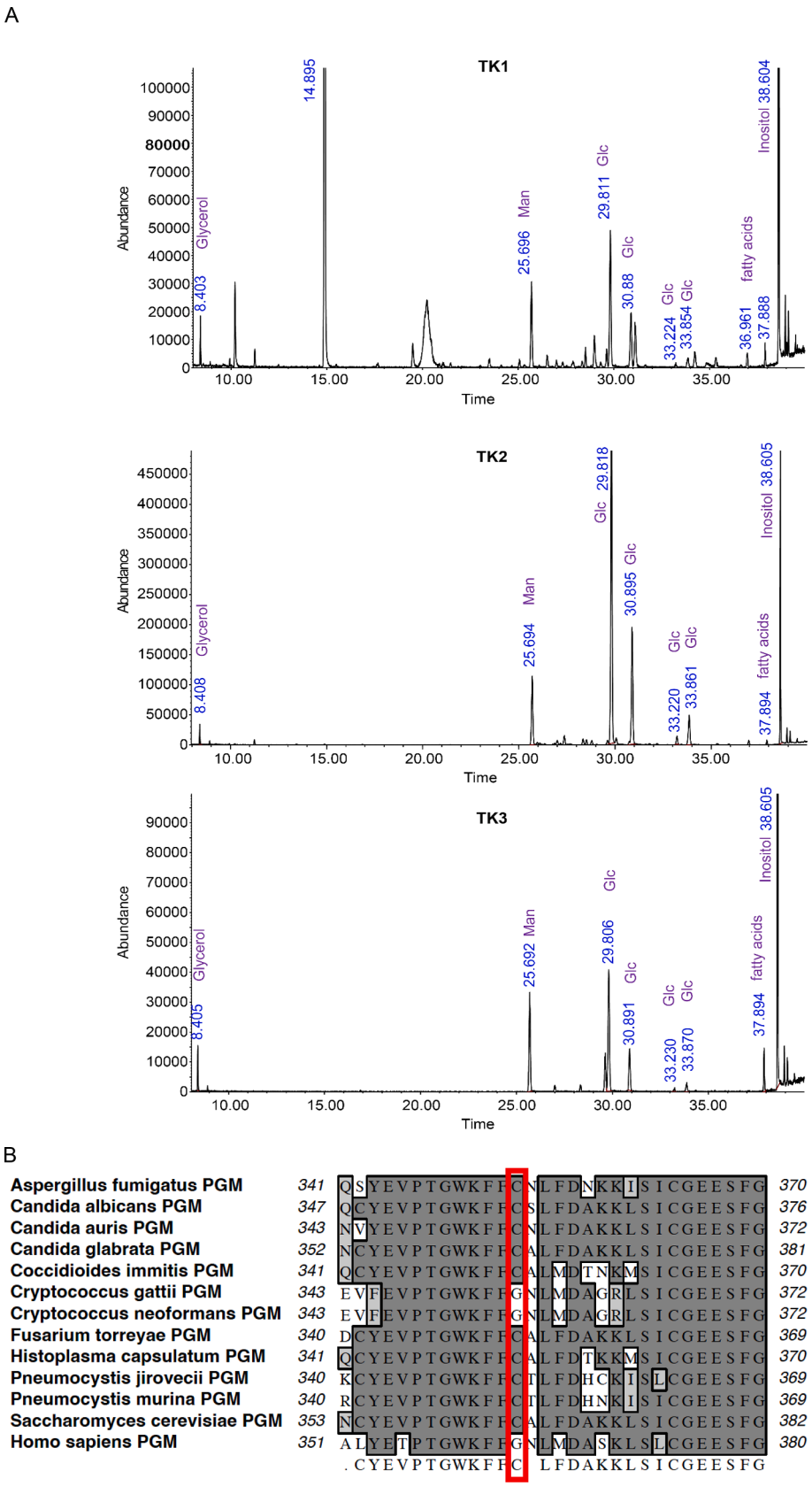

Fig. 2. (A) Total ion chromatogram for the TMS residues from the respective yeast strains. Peaks that are not labeled are non-carbohydrate components. **TK1**-BY4742 + pYES2.1/V5-His/*lacZ*, **TK2**-*pgm2*Δ + pYES2.1/V5-His/*lacZ*, **TK3**-*pgm2*Δ + *Pmpgm2*. **(B)** *S. cerevisiae,* fungal pathogen, and human PGM protein alignments. Red box containing the conserved cysteine residues of the respective PGMs. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Monosaccharide compositions and total carbohydrate by weight of the yeast cell wall pellet samples.

Glycosyl Residues	TK1 Mass (μg)	TK1 Mol %	TK2 Mass (μg)	TK2 Mol %	TK3 Mass (μg)	TK3 Mol %
Mannose (Man)	4.6	32.1	18.8	15.9	6.5	44.8
Glucose (Glc)	9.8	67.9	99.2	84.1	8.0	55.2
Sum	14.5	100	118.0	100	14.5	100
Total carbohydrate by weight %	2%	-	14.7%	-	2%	-

minimal media plates and CFUs counted. Analysis of yeast cell wall composition by gas chromatography-mass spectroscopy was performed as similar to previously described (Black et al., 2023). For protein alignments of the human PGM ortholog as well as the fungal PGM pathogens, MacVector version 18.6.4 (38) was utilized.

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Declaration of competing interest

The authors declare they have no known competing financial interests or personal relationships that may affect this work.

CRediT authorship contribution statement

Theodore J. Kottom: Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Eva M. Carmona:** Writing – review & editing, Writing – original draft. **Bernd Lepenies:** Resources. **Andrew H. Limper:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Andrew H Limper reports financial support was provided by National Institutes of Health. Theodore J Kottom reports financial support was provided by National Institutes of Health. Eva Carmona reports financial support was provided by National Institutes of Health. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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