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Extracellular targeting of *Neurospora crassa* cell wall and secreted glycoproteins by DFG-5

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ABSTRACT

The formation of a cell wall is vital for the survival and growth of a fungal cell. Fungi express members of the GH76 family of α-1,6-mannanases which play an important role in cell wall biogenesis. In this report we characterize the *Neurospora crassa* DFG-5 α-1,6-mannanase and demonstrate that it binds to the α-1,6-mannose backbone of an N-linked galactomannan found on cell wall glycoproteins. We show that DFG-5 has an enzymatic activity and provide evidence that it processes the α-1,6-mannose backbone of the N-linked galactomannan. Site-directed mutagenesis and complementation experiments show that D116 and D117 are located at the DFG-5 active site. D76 and E130, which are located in a groove on the opposite side of the protein, are also important for enzyme function. Cell wall glycoproteins co-purify with DFG-5 demonstrating a specific association between DFG-5 and cell wall glycoproteins. DFG-5 is able to discriminate between cell wall and secreted glycoproteins, and does not bind to the N-linked galactomannans present on secreted glycoproteins. DFG-5 plays a key role in targeting extracellular glycoproteins to their final destinations. By processing the galactomannans on cell wall proteins, DFG-5 targets them for cell wall incorporation by lichenin transferases. The N-linked galactomannans on secreted proteins are not processed by DFG-5, which targets these proteins for release into the extracellular medium.

1. Introduction

GH76 α-1,6-mannanases have been previously identified and characterized in gut bacteria, where they play a vital role in the digestion of fungal mannans (Cuskin et al., 2015; Thompson, Cuskin, et al., 2015; Thompson, Speciale, et al., 2015; Jones et al., 2020). They have also been found to be integral cell wall glycoproteins in fungal cell wall preparations. Multigene families of GH76 α-1,6-mannanases are encoded in fungal genomes and multiple GH76 enzymes are expressed in fungal cells creating a redundancy of GH76 α-1,6-mannanase enzyme activity (Free, 2013). Dfg5p and Dcw1p are yeast GPI-anchored cell wall GH76 family α-1,6-mannanases and have been shown to be localized to the plasma membrane and cell wall (Kitagaki et al., 2002; Spreghini et al., 2003; Maddi et al., 2012). These α-1,6-mannanases have been shown to play a vital role in cell wall biogenesis. In *Saccharomyces cerevisiae* and *Candida albicans*, the loss of GH76 enzyme activity is lethal (Spreghini et al., 2003; Kitagaki et al., 2002; Kitagaki et al., 2004; Ao et al., 2015). Characterization of *S. cerevisiae* and *C. albicans* cells in which the expression of GH76 enzymes Dfg5p and Dcw1p was minimized, either by genetic manipulation or by having a temperature-sensitive allele, demonstrates that the two enzymes are redundant and that a reduction of activity results in a weakened cell wall (Ao et al., 2015; Spreghini et al., 2003; Kitagaki et al., 2002). In *S. cerevisiae*, cells with a deficiency in Dfg5p and Dcw1p function have a rounded cell morphology, an enlarged cell size, and release of cell wall glycoproteins into the growth medium (Kitagaki et al., 2002; Gonzalez et al., 2010). In *C. albicans*, deficiency in Dfg5p and Dcw1p activity results in a cell separation mutant phenotype and the release of large numbers of cell wall glycoproteins into the growth medium (Ao et al., 2015; Mancuso et al., 2018). Mutants affected in DFG-5 activity have also been characterized in *Trichoderma atroviride* and in *A. fumigatus*. In *T. atroviride*, DFG-5 is required for hyphal morphogenesis and osmoregulation (Atanasova et al., 2021). In *A. fumigatus*, the deletion of DFG genes results in a severe growth defect and the incorporation of galactomannans into the

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cell wall is compromised (Muszkieta et al., 2019).

In *Neurospora crassa*, genetic studies confirm that DFG-5 and DCW-1 play an important role in cell wall biogenesis (Maddi et al.,2012). Deletion mutants of *dfg-5* have a spreading colonial phenotype, and *dcw-1* deletion mutants have altered cell morphology (Maddi et al., 2012). Deletion mutants lacking both are viable but have a dramatic tight colonial phenotype in which hyphal cell extension and branching are affected. The *N. crassa dfg-5, dcw-1* double mutant also releases large amounts of cell wall glycoproteins into the growth medium. A second class of mutants releasing large amounts of cell wall proteins into the medium includes the α-1,6-mannosyltransfase OCH-1, which functions to add the initial mannose residue to an N-linked galactomannan structure, and other enzymes involved in generating the galactomannan (Maddi and Free, 2010). The third class of enzymes needed for the incorporation of cell wall glycoproteins into the wall contains the GH72 lichenin transferases GEL-1, GEL-2, and GEL-5, which attach lichenin to N-linked oligosaccharides with a processed galactomannan (Kar et al., 2019). The galactomannan structure has similarities to the outer chain mannans from *S. cerevisiae* and *Candida albicans* and the outer chain galactomannans from *Schizosaccharomyces pombe* in that it has an α-1,6-mannan backbone (Gemmill and Trimble, 1999). The *N. crassa* galactomannan consists of an α-1,6-mannose backbone with galactofuranose side chains (Kar et al., 2019) (Fig. 1). The *N. crassa* full-length galactomannan with a backbone of approximately seven α-1,6-linked mannoses is much shorter than the yeast outer chain mannans (Kar et al., 2019). The GH76 α-1,6-mannanases are thought to be involved in processing the galactomannan.

N-linked galactomannans play a major role in the incorporation of *N. crassa* cell wall glycoproteins into the cell wall structure. In characterizing the N-linked glycans present on cell wall glycoproteins, we found that the glycoproteins from the *Δoch-1* mutant, which is unable to elaborate the galactomannan, were decorated with a N-acetylglucosamine 2/mannose 9 structure, as expected for a fungal high-mannose N-linked oligosaccharide (Fig. 1, A). The *Δoch-1* mutant is unable to incorporate cell wall glycoproteins into the cell wall (Maddi and Free, 2010). The *Δdfg-5, Δdcw-1* double mutant produced glycoproteins with N-linked oligosaccharides having a full-length galactomannan (Fig. 1, B) (Kar et al., 2019). These glycoproteins are released into the growth medium (Maddi et al., 2012). The *Δgel-1/Δgel-2/Δgel-5* triple mutant glycoproteins were decorated with a processed or cleaved galactomannan in which most of the structure has been removed (Fig. 1, C) (Kar et al., 2019). These results strongly suggest that the DFG-5 and DCW-1 α-1,6-mannanases cleave the full-length galactomannan to produce the processed galactomannan. The processed galactomannan was shown to be a substrate for the lichenin transferases, which attach lichenin to the processed galactomannan and thereby incorporate the glycoprotein into the cell wall (Kar et al., 2019). We presented a four-step pathway for incorporation of cell wall glycoproteins into the *N. crassa* cell wall (Kar et al., 2019; Patel and Free, 2019). First, the glycoproteins are modified by the addition of galactomannans. Second, the galactomannans are processed by DFG-5 and DCW-1. Third, lichenin transferases cleave cell wall lichenin molecules near their non-reducing ends and generate a lichenin-transferase intermediate. Fourth, the lichenin transferases transfer lichenin to the processed N-linked oligosaccharides, which generates a new glycosidic bond and incorporates the glycoproteins into the cell wall structure. Endo H treatment of the *N. crassa* cell wall releases cell wall glycoproteins, providing substantiating evidence for the incorporation of glycoproteins into the wall through their N-linked oligosaccharides (Kar et al., 2019). We have also been able to show that GEL-2, a *N. crassa* GH72 enzyme, is able to form a lichenin-enzyme intermediate, clearly demonstrating that *N. crassa* GH72 enzymes can function as lichenin transferases (Patel and Free, 2022).

In this report, we further characterize the *N. crassa* DFG-5 α-1,6-mannanase and show that it has enzymatic activity. In addition to addressing the question of how DFG-5 functions as an α-1,6-mannanase in directing glycoprotein incorporation into the cell wall, our research addresses the interesting question of how *N. crassa* cells are able to distinguish between cell wall glycoproteins and secreted glycoproteins and target each to their proper extracellular location.

2. Materials and methods

2.1. Strains and growth conditions

Wild type A and a strains (FGSC #2489 and #4200), the *his-3* mutant (FGSC #6103), the *inv* mutant (FGSC# 1856), and the deletion mutant strains for *dfg-5* (NCU03770), *dcw-1* (NCU08127), *och-1* (NCU00609),

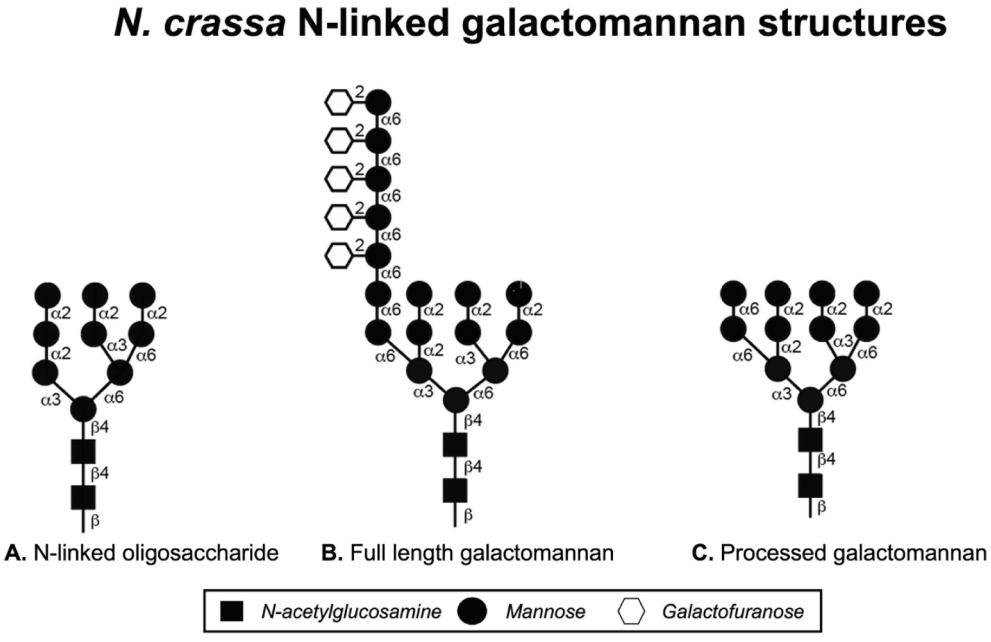

Fig. 1. Schematic drawing of the N-linked oligosaccharides found on cell wall glycoproteins from A) the Δ*och-1* mutant (no galactomannan), B) the Δ*dfg-5,* Δ*dcw-1* mutant (full-length unprocessed galactomannan), and C) the Δ*gel-1,* Δ*gel-2,* Δ*gel-5* mutant (processed galactomannan).

gam-1 (NCU01824), *acw-1* (NCU08936), *gel-1* (NCU8909), *gel-2* (NCU07253), *crh-1* (NCU05686), *gla-1* (NCU01517), and *tre-1* (NCU00943) were obtained from the Fungal Genetics Stock Center (Manhattan, KS, USA). The single deletion mutants were generated by the Neurospora Genome Project (Colot et al., 2006). The *gpig-1* temperature-sensitive mutant 34–15 was obtained from Sieler and Plamann (Seiler and Plamann, 2003). This temperature-sensitive mutant is affected in the first step of the biosynthetic pathway for glycosylphosphatidylinositol (GPI) synthesis. Strains with multiple mutations were obtained through standard genetic mating (Davis and DeSerres, 1970). Table S1 providing a listing of the various strains used in the research. These strains are available at the Fungal Genetics Stock Center. All isolates were maintained on Vogel's agar medium with 2% sucrose or 2% glucose (Davis and DeSerres, 1970). The media was supplemented with 200 μg/ml histidine when culturing the *his-3* mutant. Vogel's sorbose medium was used to isolate individual ascospore progeny from mating experiments and for the isolation of transformant isolates (Davis and DeSerres, 1970).

2.2. Cloning and tagging N. crassa DFG-5 with a HIS6 tag

Because efforts to express *N. crassa* DFG-5 in *E. coli* proved to be unsuccessful, we generated a HIS6-tagged version of the *dfg-5* gene for expression in *N. crassa*. Cloning experiments were carried out using the pBM61 vector (Margolin et al., 1997), which allows for the targeted insertion of cloned genes into the intergenic region downstream of the *his-3* locus. Transformation of the *his-3* mutant with the vector generates a wild type *his-3* allele and allows transformants to be isolated on an unsupplemented sorbose medium. A wild type version of the *dfg-5* gene was obtained by amplifying the gene from wild type genomic DNA and inserting the gene into the pBM61 vector to create pDFG5.

The *dfg-5* gene was HIS6-tagged by replacing the codons for amino acids FSDRGH at position 308–313 with a HIS6 sequence using the GeneArt® Site-Directed Mutagenesis System (Invitrogen™) to generate pDFG5HIS6. The pDFG5 and pDFG5HIS6 plasmids were purified and sequenced to verify their identity. Complementation experiments for *dfg-5* were carried out by transforming a *Δdfg-5*, *his-3* isolate with the pDFG5HIS6 using the protocol described by Margolin et al., (Margolin et al., 1997). The pDFG5HIS6 vector was also transformed in a *Δdfg-5*, *his-3*, *Δoch1* isolate to generate a transformant in which the cell wall glycoproteins were devoid of N-linked galactomannans. We transformed pDFG5HIS6 into a *his-3*, Δ*gam-1* (UDPGalactose mutase deletion) isolate and a temperature-sensitive *gpig-1* (N-acetylglucosamine transferase), *his-3* isolate to express HIS6-tagged DFG-5 in cells lacking the galactofuranose side chain associated with the galactomannan and the GPI anchor found on many glycoproteins, respectively.

2.3. Site-directed mutagenesis and complementation assays

Site-directed mutagenesis was carried out on pDFG5 and pDFG5HIS6 to replace highly conserved aspartate and glutamate residues. These mutagenesis experiments were carried out using the GeneArt® Site-Directed Mutagenesis System (Invitrogen™) and the Q5® Site-Directed Mutagenesis Kit (New England BioLabs® Inc). The plasmids with the mutant *dfg-5* genes were purified and sequenced to confirm that they contained the mutant *dfg-5* gene. To determine if mutating the conserved residues affected function, complementation experiments were carried out by transforming a *Δdfg-5*, *his-3* isolate with wild type and mutant *dfg-5* genes using the protocol described by Margolin et al., (Margolin et al., 1997). PCR reactions were used to demonstrate the presence of the transforming genes at the *his-3* locus. Complementation was assessed by inoculating five or six transformant colonies in the middle of Petri dishes containing Vogel's sucrose agar medium and examining their rate of growth during a 48-hour incubation at 30 °C. The growth rates for the five or six transformants containing each of the mutant genes were nearly identical.

2.4. PCR amplification and primers

The presence of the mutations created by site-directed mutagenesis and the presence of the sequence encoding six consecutive histidines in HIS6-tagged DFG-5 were determined by sequencing plasmids and/or by PCR amplification of the *dfg-5* gene and sequencing the PCR products. Table S2 gives the sequences of the primers used for site-directed mutagenesis, for creating the version of *dfg-5* encoding a HIS6-tagged protein, and for sequencing *dfg-5* gene constructs.

2.5. Modeling of DFG-5 protein

The amino acid sequence of the DFG-5 protein was retrieved from FungiDB (https://fungidb.org/fungidb/). The model protein structure for *N. crassa* DFG-5 was constructed with ExPASy Swiss-Model tool (https://swissmodel.expasy.org/), by using the *Bacillus circulans* and *Chaetomium thermophilum* α-1,6-mannanase proteins (PDB ID: 4BOJ and 6RY7) as templates. The sequence identity between *N. crassa* DFG-5 and *B. circulans* and *C. thermophilum* α-1,6-mannanases are 17.24% and 40%. The modeled *N. crassa* DFG-5 was visualized and analyzed using the PyMOL software program (PyMOL Version 2.0.4). *N. crassa* DFG-5 and *C. thermophilum* α-1,6-mannanase were superimposed in the PyMOL program to compare the similarity between the 3D structure. The PyMOL program was also used to prepare drawings of the *N. crassa* DFG-5 three-dimensional structure. The Clustal Omega Alignment tool was utilized to look for conserved aspartate and glutamate residues in fungal GH76 α-1,6-mannanases. The multiple expectation maximization for motif elicitation (MEME) tool was used for an analysis of the amino acid sequences surrounding N-linked oligosaccharide attachment sites.

2.6. Demonstrating that DFG-5 has enzymatic activity

To demonstrate DFG-5 enzyme activity, we needed to use glycoproteins with the full-length galactomannan as substrates. Thus, we used the cell wall glycoproteins released into the medium by the *Δdfg-5, Δdcw-1* mutant as the substrate for testing enzymatic activity. These glycoproteins were isolated as previously described, and contain a full-length galactomannan as defined by our previous glycan profiling experiments (Kar et al., 2019). Briefly, the substrate glycoproteins were precipitated with ammonium sulfate and subjected to an extensive dialysis against distilled water at 4 °C to remove any small molecular weight molecules. The substrate glycoproteins (600 μg) were incubated with (experimental) or without (control) purified HIS6-tagged DFG-5 (7 μg) in 500 ul of 10 mM sodium acetate pH5.0 buffer for 72 h at 30 °C to allow DFG-5 to cleave the galactomannans. Following the incubation, the samples were subjected to dialysis in a Slide-A-Lyzer Mini dialysis device (3.5 MWCO) (Thermo Scientific, Rockford, IL) against distilled water to allow released oligosaccharides and sugars to enter the dialysate. The dialysate was then concentrated on a speed vac and the sample was spotted on a TLC Silica gel 60 plate (Merck, Billerica, MA). The TLC plate was then developed with a mobile phase of n-butanol:acetic acid: water (2:1:1). The oligosaccharides and sugars were stained with a 1% orcinol in ethanol:sulfuric acid (70:3) stain as described by Jones et al., (Jones et al., 2020). In the absence of the purified DFG-5, the glycoprotein substrate samples released an oligosaccharide that remained at the loading site during the development of the TLC plate. The digestion of this oligosaccharide was dependent upon the presence of the purified DFG-5.

2.7. Co-purification experiments and Western blot assays

To determine if DFG-5 associates with cell wall glycoproteins, we examined the interactions between the HIS6-tagged version of DFG-5 and other glycoprotein substrates in co-purification experiments. We looked for DFG-5 interacting partners by preparing cellular extracts from cells expressing the HIS6-tagged DFG-5 and purifying the HIS6-

tagged DFG-5 with a ProBond Nickel resin using the purification protocol provided by the supplier (Life Technologies, Carlsbad, CA). The purified DFG-5 samples, along with any interacting proteins, were subjected to SDS PAGE and stained with Coomassie blue to visualize proteins co-purifying with DFG-5. We expressed HIS6-tagged DFG-5 in wild type cells, in a Δ*dfg-5* isolate, in an *Δoch-1* isolate, in a Δ*gam-1* isolate, and in a temperature-sensitive *gpig-1* strain for these co-purification experiments. We also expressed HIS6-tagged DFG-5 with D76N, D116N, D117N, D116N/D117N, and E130Q mutations in the wild type strain to determine if these mutations affected DFG-5 associations with co-purifying partners.

We used Western blot analyses with a battery of antibodies directed against cell wall and secreted glycoproteins to determine which types of glycoproteins co-purified with DFG-5. To identify DFG-5 in Western blot experiments, rabbit affinity-purified epitope antibodies directed against the peptide GGIQPGFKGIDGTAC, representing amino acids 375 to 389 from DFG-5, were prepared at Pacific Immunology (Ramona, CA). Western blots using affinity-purified epitope-specific rabbit antibodies directed against ACW-1 (NCU08936), CRH-1 (NCU05686), GEL-1 (NCU08909), and GEL-2 (NCU07253) (four major cell wall glycoproteins) were used to determine if cell wall glycoproteins co-purify with DFG-5. These antibodies were produced at Pacific Immunology using epitopes IQANGLDMEVGFPNLIWAMNMAI (ACW-1), EDIKNYGYYFA (CRH-1), YKPANEPAEC (GEL-1), and CPPKDDDWLVD (GEL-2). ACW-1, GEL-1, GEL-2, and CRH-1 have been identified as being major integral cell wall glycoproteins in proteomic studies (Bowman et al., 2006; Maddi et al., 2009; Maddi et al., 2012; Ao et al., 2016). Polyclonal rabbit antibodies directed against purified glucoamylase (GLA-1/NCU01517), invertase (INV/NCU04265), and trehalase (TRE-1/NCU00943) were used to determine if DFG-5 interacts with secreted glycoproteins (Lee and Free, 1984; Sigmund et al., 1985; White et al., 1985). Western blot experiments were carried out as previously described (Ao and Free, 2017; Kar et al., 2019). In these Western blot experiments, the antibodies were first pre-absorbed on nitrocellulose filters coated with cellular extracts from strains having deletion mutations of the genes encoding the antibody targets. We found this helped to remove small amounts of antibodies directed against non-target antigens. Presumably, the rabbits used in raising these antibodies have small amounts of antibodies directed against other fungi proteins they had encountered, which gave extraneous signals in Western blot experiments. The pre-absorption step was used to remove these non-target antibodies.

2.8. Evaluation of N-linked oligosaccharide structures on secreted glycoproteins

Glycan profiling experiments were carried out at the Complex Carbohydrate Research Center to determine the size and sugar linkages present in the N-linked oligosaccharides on a sample of previously purified alkaline phosphatase (Free and Metzenberg, 1982). The purified protein was first digested with trypsin, and the N-linked oligosaccharides were then released from the peptides by PNGaseF digestion. The N-linked oligosaccharides were permethylated and their size and composition determined by matrix-assisted laser-desorption time-of-flight mass spectrometry (MALDI-TOF/MS). Linkage analysis was carried out by subjecting the permethylated glycans to an acid hydrolysis followed by reduction and N- and O-acetylation to generate partially methylated alditol acetates (PMAAs). These were then separated and analyzed by gas chromatography-mass spectrometry. The sizes, composition, and sugar linkages found for the released N-linked oligosaccharides were used to deduce the structure of the N-linked galactomannans on the secreted proteins.

3. Results

3.1. Site-directed mutagenesis of DFG-5 defines aspartate and glutamate residues needed for enzyme activity

Aspartates and glutamates play important roles in the catalytic sites of the glycosyl hydrolase (GH) enzymes. Aspartate and glutamate residues function in the active sites of glycosyl hydrolases and are often involved in non-catalytic interactions with polysaccharide substrates. A comparison of the amino acid sequences from the GH76 enzymes encoded in fungal genomes identified aspartate and glutamate residues that are highly conserved in fungal α-1,6-mannanases. Six highly conserved aspartates and a glutamate were chosen for site-directed mutagenesis (Figure S1). We carried out a series of site-directed mutagenesis experiments on *dfg-5* in which each of these aspartate and glutamate codons was replaced by an asparagine, glutamine, or threonine codon. The mutations created were D76N, D116N, D117N, D116N/D117N, E130Q, D159Q, D230T, and D320N. Alleles with these targeted mutations were used to transform a *Δdfg-5* isolate, and the ability of the mutant allele to complement the *dfg-5* deletion was determined (Fig. 2). The HIS6-tagged DFG-5 and the D159Q, D230T, and D320N mutants all complemented the deletion. We found that three of the aspartates (D76, D116, and D117) were needed for DFG-5 activity as defined by an inability to complement the deletion mutation. The E130Q mutation showed a partial complementation phenotype with a growth rate intermediate between that of the deletion mutant and the wild type. A pair of adjacent aspartate residues have been shown to be the active sites of the *Bacillus circulans* and *Bacteroides thetaiotamicron* α-1,6-mannanases (Thompson, Cuskin, et al., 2015; Thompson, Speciale, et al., 2015; Jones et al., 2020). Based on this and the modeling experiments described below, we deduce that the D116/D117 site is the active site in DFG-5.

3.2. Modeling of N. crassa DFG-5

We carried out structural modeling experiments on DFG-5. The modeled *N. crassa* DFG-5 protein was superimposed on the *B. circulans* and *C. thermophilum* α-1,6-mannanases (PDB ID: 4BOJ and 6RY7) and we found the three proteins to be structurally similar. The structure comparison shows that the residues D116 and D117 of *N. crassa* DFG-5 lie at the same location as that of residues D124 and D125 of *B. circulans* and D134 and D135 of the *C. thermophilum* α-1,6-mannanases in the long groove on one face of the enzyme (Fig. 3, A). Based on the position of the aspartate residues and the reaction mechanism worked out for the *B. circulans* and *B. thetaiotamicron* α-1,6-mannanases, D116 and D117 represent the catalytic active site of *N. crassa* DFG-5 (Thompson, Speciale, et al., 2015; Jones et al., 2020). Complementation experiments show that the D76 and E130 residues are important for the activity of DFG-5. Interestingly, D76 and E130 are located in a second groove found on the surface of DFG-5 opposite from the active site (Fig. 3, B). The specific role of D76 and E130 residues for the DFG-5 enzyme activity is unknown.

3.3. Characterization of DFG-5 enzymatic activity

To better understand DFG-5 α-1,6-mannanase activity and definitively demonstrate that it has an enzymatic activity, we used a nickel column to purify HIS6-tagged DFG-5 from wild type and *Δoch-1* mutant isolates for our characterization of DFG-5 enzymatic activity. As shown in the following sections, purification of HIS6-tagged DFG-5 from *Δoch-1* cells allows us to purify DFG-5 free of co-purifying cell wall glycoproteins. To provide a substrate for the assay, we used cell wall glycoproteins that had been released into the growth medium by a *Δdfg-5, Δdcw-1* double mutant. These cell wall glycoproteins have been previously shown to contain a full-length N-linked galactomannan (Fig. 1) (Kar et al., 2019). To assay for enzymatic activity HIS6-tagged DFG-5 was purified from the Δ*och-1* isolate, added to the substrate cell wall

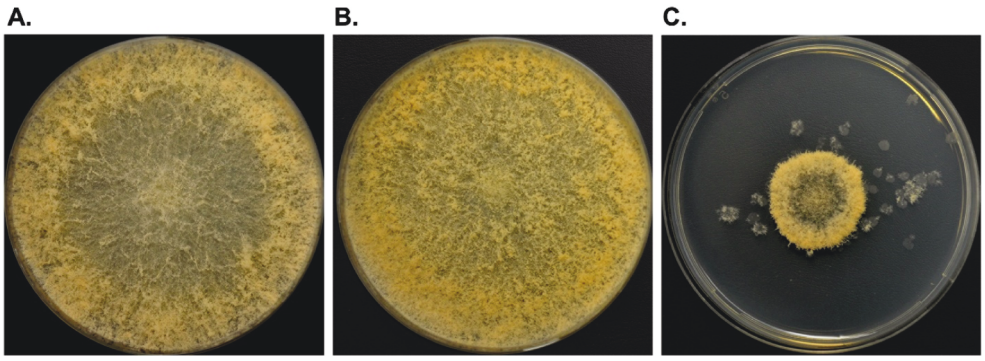

Fig. 2. Site-directed mutagenesis of *dfg-5*. Site-directed mutagenesis was used to create the mutant genes used for complementation experiments. Representative complementation experiments are shown for A) complementation by the wild type *dfg-5* gene, B) complementation by the *dfg-5* D159Q mutant gene, and C) lack of complementation by the *dfg-5* D116N/D117N mutant gene.

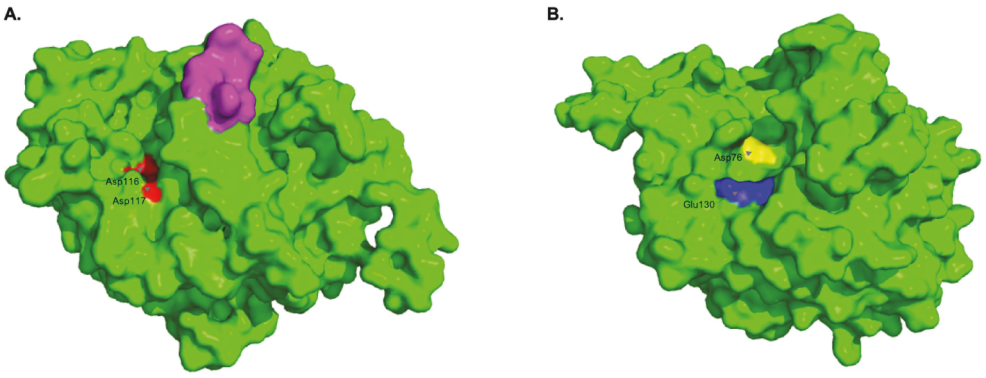

Fig. 3. Modeling of DFG-5. A model of the DFG-5 α-1,6-mannanase was generated using the Expasy Swiss-Model tool. A) The active site D116/D117 (shown in red) lies in a long groove. The amino acids shown in magenta were replaced with the HIS6 tag in the HIS6-tagged DFG-5 enzyme. B) The amino acid residues D76 (yellow) and E130 (blue) lie close to each other in a groove on the other side of DFG-5. The image in A has been rotated approximately 180° (left to right) to obtain the image in B. A loop with amino acids 360 to 387 is seen at the lower right in image A and at the upper left in image B. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

glycoproteins, and incubated at 30 °C for 72 h. We found that during our assay the substrate glycoproteins in the control sample (without DFG-5) release a large oligosaccharide from their post-translational modifications that remains at the loading spot in our TLC assay system (Figure S2). This large oligosaccharide must come from the N-linked oligosaccharides associated with the substrate glycoproteins. We surmise that the cell wall glycoprotein substrate must contain one or more hydrolases that act to release of the oligosaccharide from the substrate glycoproteins. When purified DFG-5 is included in the reaction, this oligosaccharide is digested (Figure S2). We conclude that the digestion of the oligosaccharide is dependent upon the presence of the purified DFG-5.

To further demonstrate that the digestion of the oligosaccharide depends on DFG-5 enzymatic activity, the assay was carried out with purified HIS6-tagged DFG-5 containing site-directed mutations at D76N, D116N/D117N, and E130Q. The wild type DFG-5 was able to participate in the digestion the oligosaccharide, but the D116N/D117N mutation at the active site and the D76N and E130Q mutations abolish the enzymatic activity of DFG-5 (Fig. 4). We conclude that DFG-5 has an enzymatic activity that is required for the digestion of the oligosaccharide and that the non-complementing mutant enzymes have lost enzymatic activity. We note that the monosaccharides and/or disaccharides that might be expected from the digestion of the oligosaccharide are not observed in the TLC analysis, and we ascribe this to the presence of the cell wall hydrolases and oxidases found in the substrate glycoprotein sample.

Although we have not definitely defined the structure of the oligosaccharide, the results clearly demonstrate that DFG-5 is participating in the digestion of a glycoprotein-associated oligosaccharide that is derived from glycoproteins with full-length N-linked galactomannans. Although further experimentation is needed to fully characterize the reaction(s)

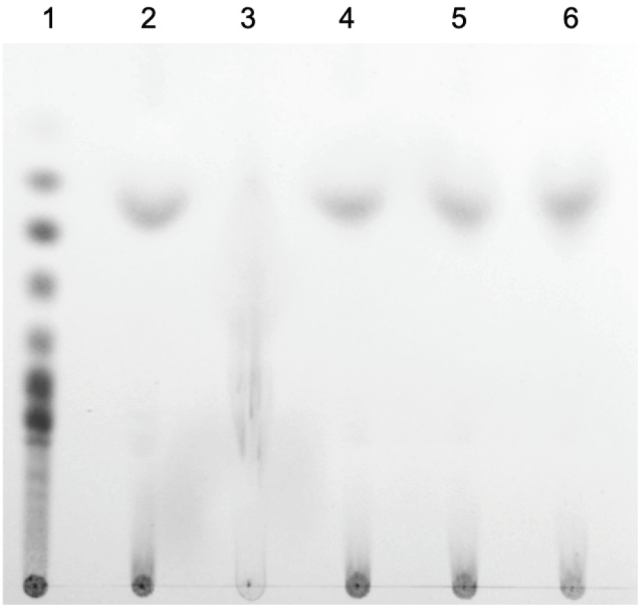

Fig. 4. TLC assay for DFG-5 enzyme activity. Lane 1 contains maltodextrin, which serves as a control and size marker. Substrate cell wall glycoproteins from a Δ*dfg-5*, Δ*dcw-1* mutant were incubated without (lane 2) or with (lane 3) purified HIS6-tagged DFG-5. Lanes 4, 5, and 6 contain mutant DFG-5 s with D76N, D116N/D117N, and E130Q mutations respectively. DFG-5 participates in the digestion of the oligosaccharide seen at the loading position (lane 3).

seen in our TLC assay, the results strongly suggest that DFG-5 α-1,6-mannanase is able to recognize and digest the α-1,6-mannan backbone of the N-linked galactomannan. This conclusion is further substantiated by our previous characterization of the N-linked glycans found on cell wall glycoproteins from the Δ*och-1* mutant, the Δ*dfg-5*, Δ*dcw-1* double mutant, and the Δ*gel-1*, Δ*gel-2*, Δ*gel-5* triple mutant lacking lichenin transferase activity (Fig. 1) (Kar et al., 2019). In these studies, we found that the difference between the N-linked glycan from the Δ*dfg-5,* Δ*dcw-1* double mutant which lacks the α-1,6-mannanases (the full-length unprocessed galactomannan) and the N-linked glycan from the Δ*gel-1,* Δ*gel-2,* Δ*gel-5* triple mutant which has the α-1,6-mannanases but lacks the lichenin transferases needed to incorporate glycoproteins into the wall (the processed galactomannan) was the loss of the terminal elements of the galactomannan.

3.4. DFG-5 binds cell wall glycoproteins

To determine if DFG-5 forms associations with other proteins, we generated a HIS6-tagged version of DFG-5 by replacing amino acids 308 to 313 with a HIS6 tag, as described in the Materials and Methods section. The location of the HIS6 tag in the three-dimensional structure of DFG-5 is shown in Fig. 3. The HIS6-tagged DFG-5 was able to fully complement the *dfg-5* deletion indicating that the tagged protein was enzymatically active. When we purified the HIS6-tagged DFG-5 from cellular extracts on a nickel column, we found that a large number of glycoproteins co-purified with the HIS6-tagged DFG-5 (Fig. 5, A). DFG-5 is a cell wall glycoprotein, so the DFG-5 being purified in our procedure must represent DFG-5 that is *in transit* in the secretory system and DFG-5 that is found in the cell wall space but not yet covalently incorporated into the insoluble cell wall. Using the co-purification protocol with extracts from the *Δdfg-5, his-3* mutant (the parental strain used for the transformation experiments with the HIS6-tagged DFG-5 vector) demonstrated that these glycoproteins are specifically co-purifying with DFG-5 (Fig. 5, A). Western blot experiments with an epitope antibody directed against DFG-5 demonstrated that the major band of proteins in our co-purification is the HIS6-tagged DFG-5 with a molecular weight of 72 kDa (Fig. 5, B). The predicted molecular weight of DFG-5, assuming loss of the signal peptide, is approximately 50 kDa. This suggests that glycosylation adds approximately 22 kDa to the molecular weight. DFG-5 has six possible N-linked oligosaccharide addition sites and 65 serines and threonines that could be O-glycosylated.

3.5. Binding of cell wall glycoproteins to DFG-5 requires the presence of N-linked galactomannans

With a large number of glycoproteins co-purifying with DFG-5, we asked if the co-purification of these glycoproteins was affected in the mutant DFG-5 proteins we generated by site-directed mutagenesis. We therefore generated D76N, D116N, D117N, D116N/D117N, and E130Q mutations of the HIS6-tagged DFG-5, expressed these in Δ*dfg-5* cells, and purified the mutant HIS6-tagged DFG-5 proteins on a nickel column (Fig. 5). We found that the D116N/D117N mutations in the active site do not abolish the ability of DFG-5 to associate with the co-purifying glycoproteins. The D76N and E130Q mutations may have some effect on the ability of DFG-5 to associate with its co-purifying partners, but do not fully abolish these interactions.

To determine whether the presence of an N-linked galactomannan on the co-purifying proteins was required for co-purification, we expressed the HIS6-tagged DFG-5 in an *Δoch-1* mutant, which is unable to elaborate the N-linked galactomannan, and purified the protein on a nickel column. The results of the co-purification experiment demonstrate that the co-purifying glycoproteins require the presence of the galactomannan in order to co-purify with DFG-5 (Fig. 6). Fig. 6 also demonstrates that a highly purified HIS6-tagged DFG-5 (free from the co-purifying glycoproteins) can be isolated by using the Δ*och-1* mutant for the production of the enzyme.

3.6. Binding of cell wall glycoproteins to DFG-5 does not require the galactomannan galactofuranose side chain or the presence of a GPI anchor

To further characterize the associations between DFG-5 and its co-purifying glycoproteins, we expressed the HIS6-tagged DFG-5 in a Δ*gam-1* mutant. GAM-1 (UDP-Galactose mutase) catalyzes the conversion of UDP-Galactose to UDP-Galactofuranose. GAM-1 is required to generate the galactofuranose found as the galactofuranose side chain of the N-linked galactomannan (Fig. 1). A co-purification experiment from HIS6-tagged DFG-5 expressed in the Δ*gam-1* mutant shows that the loss of the galactofuranose side chains from the N-linked galactomannan does not significantly affect the ability of the co-purifying glycoproteins to associate with DFG-5 (Fig. 7, A). We conclude that DFG-5 must be recognizing the α-1,6-mannan backbone of the N-linked galactomannan.

Based on a report suggesting that *C. thermophilum* DFG-5 might bind the GPI anchor structure present on many cell wall proteins (Vogt et al., 2020), we decided to determine if the presence or absence of a GPI anchor affects the ability of co-purifying glycoproteins to form associations with DFG-5. The HIS6-tagged DFG-5 was therefore expressed in a temperature-sensitive *gpig-1* mutant which is affected in the first step in

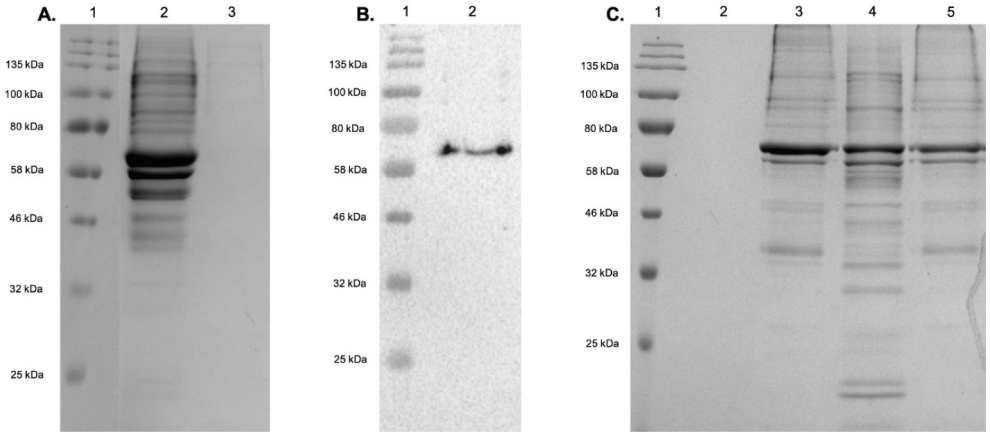

Fig. 5. HIS6-tagged DFG-5 binds to a number of *N. crassa* glycoproteins. A) An image of a Coomassie-stained gel of the glycoproteins that co-purify with HIS6-tagged DFG-5 (lane 2). Lane 1 contains molecular weight markers and lane 3 is a control co-purification experiment from cells lacking the HIS6-tagged DFG-5 to demonstrate that the co-purifying proteins are associating with DFG-5. B) A Western blot image of the HIS6-tagged DFG-5 co-purification using anti-DFG-5 antibodies showing that the major band in the co-purification is HIS6-tagged DFG-5. C) A Coomassie-stained gel showing DFG-5 co-purification experiments. The Coomassie-stained gel contains molecular weight markers (lane 1), a control co-purification from a Δ*dfg-5* isolate (lane 2), glycoproteins co-purifying from the D76N mutant DFG-5 (lane 3), glycoproteins co-purifying from the D116N/D117N mutant DFG-5 (lane 4), and glycoproteins co-purifying from the E130Q mutant DFG-5 (Lane 5).

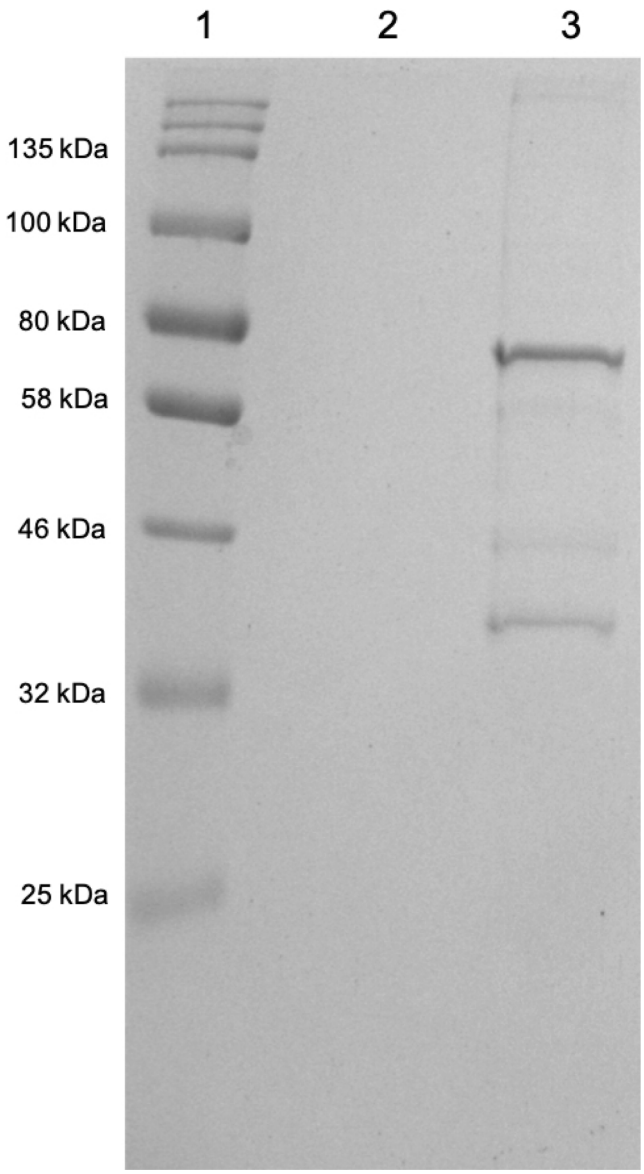

Fig. 6. DFG-5 recognizes N-linked galactomannans. A Coomassie-stained gel of the glycoproteins that co-purify with HIS6-tagged DFG-5 expressed in an Δ*och-1* isolate is shown. Lane 1 contains molecular weight markers. Lane 2 is a control co-purification from a non-transformed Δ*och-1* isolate. Lane 3 shows the glycoproteins co-purifying with a HIS6-tagged DFG-5 being expressed in the *Δoch-1* mutant.

the biosynthetic pathway for GPI anchor formation. We found that there were no differences in the pattern of DFG-5 co-purifying glycoproteins when the mutant was grown at the permissive temperature and after a four-hour shift to the restrictive temperature (Fig. 7, B). We conclude that the GPI anchor is not involved in the associations between the *N. crassa* DFG-5 and its co-purifying partners.

3.7. DFG-5 is able to discriminate between cell wall glycoproteins and secreted glycoproteins

To determine whether DFG-5 specifically recognizes cell wall glycoproteins, a number of Western blot experiments were done using antibodies directed against known cell wall glycoproteins and glycoproteins that are secreted into the growth medium. HIS6-tagged DFG-5 was expressed in a *Δdfg-5* isolate, purified on the nickel column, and the co-purifying proteins subjected to Western blot analyses with affinity-purified epitope antibodies for ACW-1 (NCU08936), GEL-1 (NCU08909), GEL-2 (NCU07253) and CRH-1(NCU05686). ACW-1, GEL-1, GEL-2, and CRH-1 have all been shown to be major cell wall glycoproteins (Bowman et al., 2006; Maddi et al., 2009). Our results demonstrate that each of these cell wall glycoproteins co-purified with the HIS6-tagged DFG-5 (Fig. 8). We conclude that DFG-5 is able to interact with a battery of major cell wall glycoproteins. The fact that we don't see these interactions when the glycoproteins are produced without the N-linked galactomannan (Fig. 6) indicates that these interactions require the presence of the N-linked galactomannan.

To further characterize DFG-5 interactions with other glycoproteins, we also carried out a series of Western blot experiments looking for interactions with secreted glycoproteins. In particular, we assessed the ability of DFG-5 to bind to invertase, glucoamylase, and trehalase, three well-characterized secreted glycoproteins. For these experiments, the HIS6-tagged DFG-5 was expressed in the *Δdfg-5* isolate that had been grown in the presence of sucrose, amylose, or trehalose to induce expression of these proteins. Cellular extracts were prepared and subjected to Western blot analysis with antibodies directed against the secreted glycoproteins (Lee and Free, 1984; White et al., 1985; Sigmund et al., 1985). The results of these Western blot experiments demonstrate that invertase, glucoamylase, and trehalase do not co-purify with DFG-5 (Fig. 9). We conclude that DFG-5 is able to discriminate between glycoproteins that are targeted for secretion and glycoproteins targeted for incorporation into the cell wall.

3.8. Secreted extracellular proteins have an unprocessed galactomannan

With cell wall glycoproteins interacting with DFG-5 and secreted glycoproteins not being able to associate with the α-1,6-mannanase, we decided to examine the N-linked oligosaccharides present on secreted glycoproteins. Alkaline phosphatase (PHO-2/NCU01376) is a secreted glycoprotein whose expression is highly induced when *N. crassa* cells are starved for phosphate in slightly alkaline culture conditions (Burton and Metzenberg, 1974; Toh and Ishikawa, 1971). A highly purified sample of alkaline phosphatase was available from previous studies (Free and Metzenberg, 1982), and was subjected to glycan profiling and linkage analysis experiments at the Complex Carbohydrate Research Center (Fig. 10). The purified enzyme contained N-linked oligosaccharides with sizes up to 25 sugars, and linkage analysis shows the presence of 2,6-linked mannose and terminal galactofuranose (Fig. 10). The 2,6-linked mannose and terminal galactofuranose are characteristic of the galactomannan structure and not found in basic N-linked oligosaccharide structure (Fig. 1). These results demonstrate that the secreted alkaline phosphatase contains full-length N-linked galactomannan. Considering these results in conjunction with the experiments showing DFG-5 interacts with cell wall glycoproteins containing full-length N-linked galactomannans, we conclude that the ability of DFG-5 to recognize and interact with cell wall glycoproteins requires more than the presence of the galactomannan. Our results suggest that additional features, beyond the presence of the galactomannan substrate, are being recognized by DFG-5 and allow the α-1,6-mannanase to specifically process the galactomannans on cell wall glycoproteins to target them for incorporation into the cell wall. Glycoproteins not recognized by DFG-5 retain their full-length N-linked galactomannan and, lacking a processed galactomannan, are targeted for secretion.

3.9. Analysis of N-linked oligosaccharide addition sites in cell wall and secreted extracellular glycoproteins

To determine if the amino acid sequence flanking N-linked addition sites might represent a possible recognition site for DFG-5 and allow DFG-5 to discriminate between cell wall and secreted glycoproteins, we did a MEME analysis of the amino acid sequences flanking N-linked oligosaccharide addition sites in 38 *N. crassa* cell wall proteins and 12 secreted proteins. A list of the proteins chosen for this analysis is found

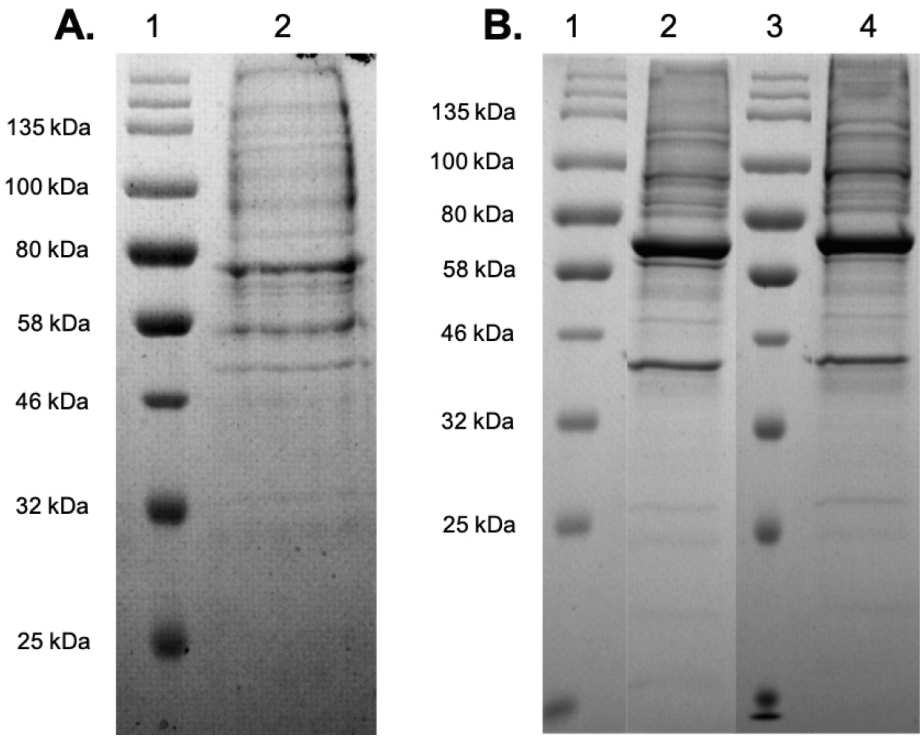

Fig. 7. DFG-5 binds to glycoproteins with galactomannans lacking galactofuranose side chains and to glycoproteins lacking GPI anchors. A) A Coomassie-stained gel containing molecular weight markers (lane 1) and DFG-5 co-purifying glycoproteins from a Δ*gam-1* transformant, which is unable to generate galactofuranose side chains to the N-linked galactomannan (lane 2). B) A Coomassie-stained gel is shown with DFG-5 co-purifying glycoproteins from a temperature-sensitive *gpig-1* mutant grown at the permissive temperature (lane 2) and at the restrictive temperature (lane 4). Lanes 1 and 3 contain molecular weight markers.

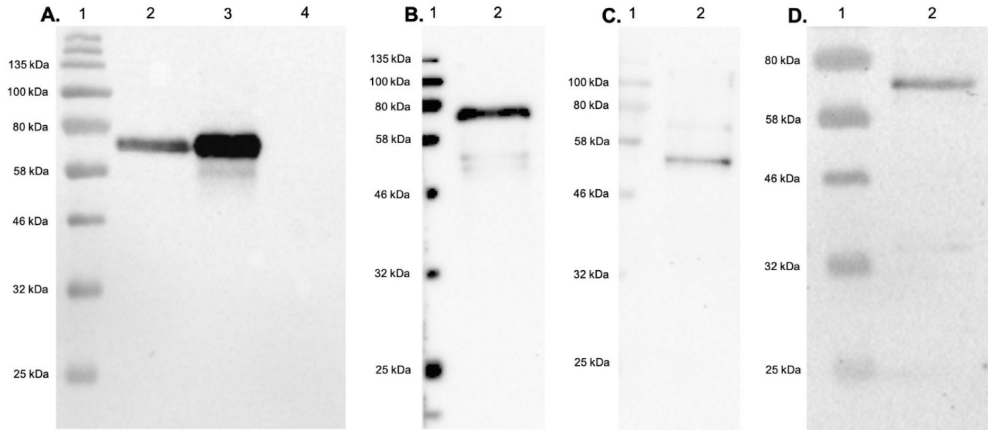

Fig. 8. DFG-5 specifically binds to cell wall glycoproteins. Western blot analyses of the glycoproteins co-purifying with HIS6-tagged DFG-5. The glycoproteins co-purifying with HIS6-tagged DFG-5 were separated by SDS PAGE and subjected to Western blot analysis with antibodies directed against cell wall glycoproteins. A) A Western blot using antibodies directed against the major cell wall protein ACW-1. Lane 1 contains molecular weight markers. Lane 2 contains HIS6-tagged DFG-5 co-purifying glycoproteins. Lane 3 contains a sample of the cellular extract used for the co-purification. Lane 4 is a control co-purification from an isolate without the HIS6-tagged DFG-5. B), C), and D) are Western blots of HIS6-tagged DFG-5 co-purifying glycoproteins using antibodies directed against GEL-1 (B), GEL-2 (C), and CRH-1 (D), three known cell wall glycoproteins. These Western blots contain molecular weight markers in lane 1 and co-purifying glycoproteins in lane 2.

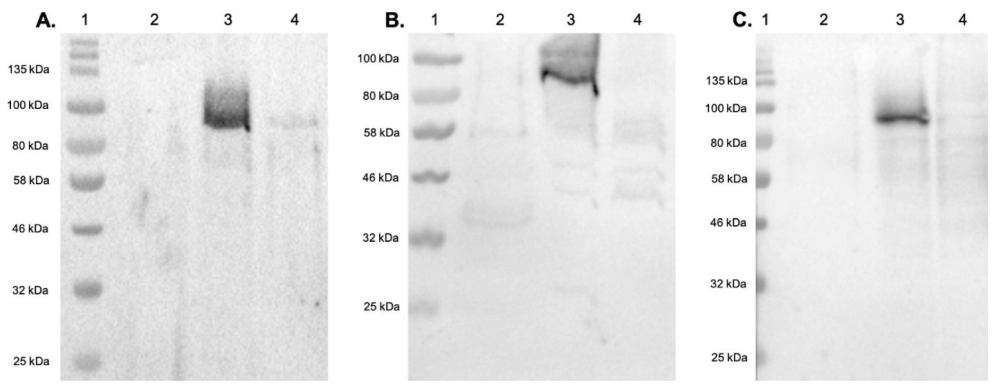

Fig. 9. HIS6-tagged DFG-5 does not recognize secreted glycoproteins. A), B), and C) are Western blot experiments using antibodies directed against invertase (A), glucoamylase (B), and trehalase (C). In all three Western blots lane 1 contains MW marker, lane 2 contains HIS6-tagged DFG-5 co-purifying proteins, and lane 3 contains the cellular extract used for the co-purification. Lane 4 contains cellular extracts from an invertase mutant (A), glucoamylase deletion mutant (B) and a trehalase deletion mutant (C) and is included to demonstrate the specificity of the antibodies.

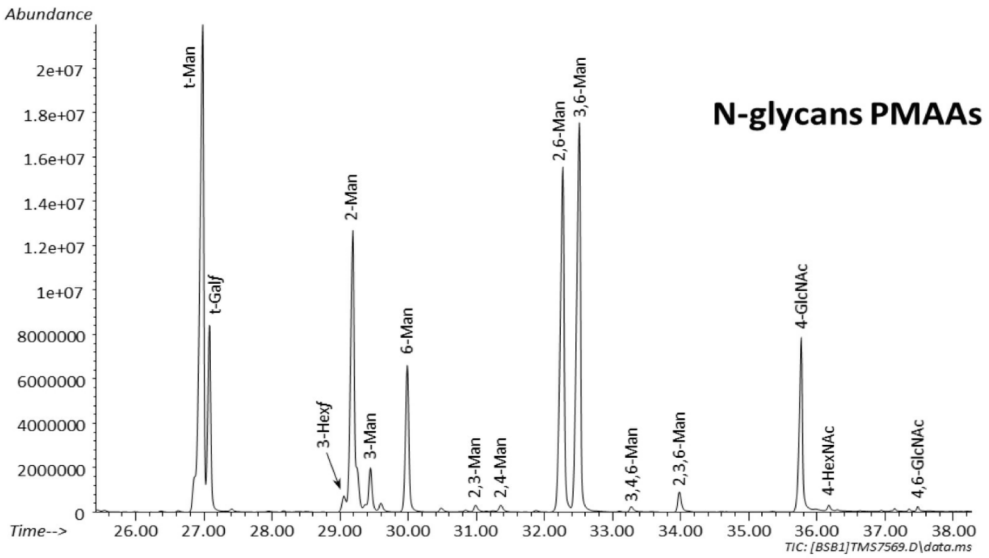

Fig. 10. Linkage analysis of N-linked galactomannan samples from a secreted glycoprotein. A linkage analysis for the N-linked oligosaccharides released by PNGaseF treatment of a highly purified sample of alkaline phosphatase, a secreted glycoprotein. The N-linked oligosaccharides contain 2,6-linked mannose residues and terminal galactofuranose residues, which are diagnostic for the presence of galactomannans.

in the Figure legend of Figure S3. The cell wall proteins were chosen from previous cell wall proteomic analyses (Maddi, Bowman, and Free, 2009; Maddi and Free, 2010; Ao et al., 2016; Bowman et al., 2006; Maddi et al., 2012). The secreted proteins were chosen from the literature and included secreted hydrolases known to function in nutrient acquisition. The MEME analysis is shown in Figure S3. We were unable to identify any clear-cut sequence biases or amino acid consensus sequences which might distinguish between cell wall and secreted glycoproteins. We hypothesize that DFG-5 must be using some more subtle structural elements to differentiate between the two groups of glycoproteins. A similar situation exists for the mammalian enzyme N-acetylglucosamine-1-phosphate transferase, which discriminates between soluble glycoproteins being targeted to the lysosome and those being targeted for secretion, but the basis of its ability to discriminate between these groups of glycoproteins remains unknown (Coutinho et al., 2012).

4. Discussion

The GH76 family of α-1,6-mannanases have been shown to be important cell wall biosynthetic enzymes in *S. cerevisiae*, *C. albicans*, *A. fumigatus*, *T. atroviride*, and *N. crassa* (Kitagaki et al., 2002; Kitagaki, Ito, and Shimoi, 2004; Spreghini et al., 2003; Maddi et al., 2012; Ao et al., 2015; Atanasova et al., 2021; Muszkieta et al., 2019). In these organisms, at least two genes from the GH76 multigene family are expressed during vegetative growth providing a redundancy of α-1,6-mannanase activity. Loss of GH76 enzymes results in the release of cell wall glycoproteins into the growth medium (Spreghini et al., 2003; Maddi et al., 2012; Ao et al., 2015; Gonzalez et al., 2010). In *S. cerevisiae* and *C. albicans*, deletion of both Dfg-5p and Dcw-1p is lethal, but in *N. crassa* the *Δdfg-5, Δdcw-1* double deletion mutant is viable and has a tight colonial growth morphology (Kitagaki et al., 2002; Spreghini et al., 2003; Maddi et al., 2012; Ao et al., 2015). Analyses of the *N crassa Δdfg-5* mutant, the *Δdcw-1* mutant, and the *Δdfg-5, Δdcw-1* double mutant indicate that there are some slight differences in the repertoire of proteins released into the medium by *Δdfg-5* and *Δdcw-1*, but the enzymes are largely redundant (Maddi et al., 2012). Based on genetic and biochemical analyses of *N. crassa* mutants affected in incorporating cell wall glycoproteins into the cell wall, a model describing the process has been proposed (Kar et al., 2019; Patel and Free, 2019). The model posits that DFG-5 and DCW-1 cleave N-linked galactomannans on cell wall glycoproteins to provide a substrate for GH72 family lichenin transferases, which attach lichenin (a mixed β-1,3-/β-1,4-glucan) to the processed N-linked galactomannan and thereby attach the glycoproteins to the cell wall matrix. The experiments described in this report characterizing the enzymatic activity of DFG-5 and its interactions with glycoproteins strongly support this model.

In this report, we provide evidence that *N. crassa* DFG-5 α-1,6-mannanase carries out the proposed enzymatic cleavage of N-linked galactomannans. To the best of our knowledge, this is the first report demonstrating an enzymatic activity for a fungal DFG-5 α-1,6-mannanase. Using cell wall glycoproteins from a *Δdfg-5,Δdcw-1* double mutant as a substrate, we found that a purified DFG-5 participated in the digestion of an oligosaccharide that is released from the substrate glycoproteins (Fig. 4). Mutations in DFG-5 that render DFG-5 inactive, as defined by a complementation assay, abolish its enzymatic activity (Fig. 4). The evidence we present suggests that the oligosaccharide includes elements of the galactomannan.

Although we haven't fully characterized the structure of the oligosaccharide or how it is released, our data indicates that hydrolases present in the substrate cell wall glycoproteins are responsible for its formation. Because DFG-5 recognizes the full-length galactoamannan on cell wall glycoproteins, but not on secreted glycoproteins (Sections 3.7 and 3.8), we think that the context in which DFG-5 sees the galactomannan is important. We think that during our TLC assay procedure, DFG-5 probably recognizes the galactomannans present on the substrate glycoproteins and cleaves their α-1,6-mannose backbone while the galactomannans are still attached to the glycoproteins (in their native conformational state). We hypothesize that the released fragments are then further degraded by the hydrolases and oxidases found in the substrate glycoproteins. In the absence of DFG-5, we surmise that the other hydrolases and oxidases can release the galactomannan but not further digest it.

We demonstrated that DFG-5 recognizes and binds N-linked galactomannan displayed on cell wall glycoproteins (Fig. 5). This association between the galactomannan and DFG-5 was not dependent upon the presence of the galactofuranose side chain found on the galactomannan, suggesting that the interactions between DFG-5 α-1,6-mannanase and the galactomannan was specific to the α-1,6-mannan backbone (Fig. 6). Our previous results characterizing the glycans present on the glycoproteins from a *Δdfg-5*, *Δdcw-1* mutant lacking α-mannanase activity and the glycans present on the glycoproteins from a lichenin transferase mutant provides additional evidence for DFG-5

acting as an α-1,6-mannanase on the N-linked galactomannan substrate (Kar et al., 2019). The difference between these glycans is that the processed galactomannan found on the cell wall glycoproteins from the lichenin transferase mutant (which has DFG-5 and DCW-1 galactomannan processing activity but is unable to incorporate the glycoproteins into the cell wall) lacked almost all of the galactomannan structure (Fig. 1). This, along with the studies detailed in this report, provides strong evidence for DFG-5 having an α-1,6-mannanase activity which processes the galactomannans present on cell wall glycoproteins. Our experiments demonstrating that DFG-5 has enzymatic activity provide substantiating evidence in favor of our proposed model for the incorporation of *N. crassa* glycoproteins into the cell wall.

Characterization of the DFG-5 α-1,6-mannanase showed that the enzyme contains two adjacent aspartate residues at its active site. Many glycosyl hydrolase enzymes, including those from the GH76 family, use an acid/base type of reaction mechanism in which aspartate and glutamate residues play an enzymatic role. In DFG-5, D116 and D117 are positioned to facilitate such a reaction mechanism. They are located in the enzyme model (Fig. 3) at the same position as the active site aspartates in the *B. circulans* and *B. thetaiotamicron* GH76 enzymes (Thompson, Cuskin, et al., 2015; Thompson, Speciale, et al., 2015; Jones et al., 2020). We also found that D76 and E130 were important for DFG-5 function. These two amino acids are located close to each other in a groove on the side of DFG-5 that is opposite the active site. D76 is found in the context of a highly conserved DYWW sequence and E130 in a highly conserved AAE sequence (Figure S1). We currently have no information about why the D76N and E130Q mutations affect DFG-5 activity, but our results and their placement within a highly conserved sequence suggests that the groove in which they are located (Fig. 3) plays an important role in DFG-5 function. The interactions we see between DFG-5 and its co-purifying cell wall glycoproteins might be subtlety affected, but not totally disrupted, when these two amino acids are changed. The fact that we purify similar amounts of mutant and wild type DFG-5 from cell extracts (Fig. 5) suggests that the mutations do not render DFG-5 unstable and susceptible to proteolysis. Further experimentation will be needed to define the role of the D76 and E130 groove in DFG-5.

Co-purification experiments demonstrated DFG-5 forms stable interactions with a large number of cell wall glycoproteins (Figs. 5, 7, and 8). Mutations at the active site, at D76, and at E130 do not seem to dramatically affect the ability of DFG-5 to form such interactions. However, expression of DFG-5 in the *Δoch-1* mutant demonstrates that the presence of an N-linked galactomannan is required for these glycoprotein interactions. This indicates that the binding of the galactomannan substrate is required for the interactions between DFG-5 and its co-purification partners, but that the active site aspartate residues are not required for binding. Most importantly, our results showed that DFG-5 specifically associates with cell wall glycoproteins. Using a Western blot assay, we found that DFG-5 specifically associates with ACW-1, CRH-1, GEL-1 and GEL-2, four well-known cell wall glycoproteins (Fig. 8). Western blot assays for glucoamylase, invertase, and trehalase, three well-known *N. crassa* secreted glycoproteins, demonstrated that DFG-5 does not form associations with secreted glycoproteins (Fig. 9). DFG-5 and its co-purifying cell wall glycoproteins all become covalently attached to the cell wall matrix soon after they are released into the cell wall space. The DFG-5 and its co-purifying glycoproteins that we observe in our experiments are present in the cell extract and must therefore represent “in transit” glycoproteins traveling through the secretory pathway and/or those recently released into the cell wall space but not yet incorporated into the wall. Galactomannan addition must occur after the co-translational addition of the N-linked oligosaccharide and is likely to occur in the Golgi apparatus. We hypothesize that DFG-5 (which is a GPI-anchored glycoprotein) and the co-purifying glycoproteins (many of which are GPI-anchored) travel together through the secretory pathway and interact with each other during the later stages of the secretory pathway and in the cell wall space.

A comparison of our results with those in a recent detailed characterization of the DFG family proteins from *A. fumigatus* is informative (Muszkieta et al., 2019). Six active DFG enzymes were identified in *A. fumigatus*, with DFG3 being the most important. Deletion of DFG3 resulted in a reduced growth rate and a hyperbranching hyphal morphology, while the sextuple deletion of the active DFG enzymes resulted in a severe growth phenotype and morphological defect. The sextuple deletion mutant was unable to incorporate galactomannan into the cell wall. Our *N. crassa* findings have a number of things in common with those from *A. fumigatus*. First, both studies provide compelling evidence for DFG-5 being involved in the incorporation of a galactomannan into the cell wall structure. In *N. crassa*, the galactomannans seem to be largely restricted to N-linked oligosaccharides and their incorporation into the wall attaches glycoproteins to the wall. In *A. fumigatus*, all of the galactomannans are thought to be found as free galactomannans or as GPI-anchored membrane-associated galactomannans, and these galactomannans become cell wall elements (Li et al., 2018). Another area of commonality between the two studies is the presence of α-1,6-linked mannoses within the backbone of the target molecule. While the *N. crassa* galactomannan has a relatively short α-1,6-mannose backbone with galactofuranose side chains, the *A. fumigatus* galactomannans are larger and have a backbone containing α-1,2-mannotetrose repeating subunits attached via α-1,6-mannose linkages with side chains of four or five galactofuranose residues (Latge et al., 1994). In our study, we found that the α-1,6-linked mannose backbone was the critical element for recognition of the galactomannan by DFG-5. Interestingly, the *A. fumigatus* DFG3 was shown to be able to complement the *S. cerevisiae* DFG5 mutant (Muszkieta et al., 2019). *S. cerevisiae* does not have a galactomannan similar to that found in A. *fumigatus*, but it does have outer chain mannans with an α-1,6-mannose backbone that might represent a substrate for the enzyme.

The most novel aspect of our report is that DFG-5 plays a central role in the extracellular targeting of cell wall and secreted glycoproteins. We demonstrated that secreted glycoproteins have full-length N-linked galactomannans (Fig. 10). This indicates that cell wall glycoproteins and secreted glycoproteins are both modified by the addition of the full-length galactomannan while traveling through the secretory pathway. Our results show that DFG-5 is able to specifically recognize the cell wall glycoproteins and cleave their N-linked galactomannans to generate processed galactomannans in which most of the galactomannan structure is removed from the N-linked oligosaccharide (Fig. 1). Our results indicate that the ability of DFG-5 to discriminate between cell wall and secreted glycoproteins requires that DFG-5 recognition of cell wall glycoproteins is dependent upon the presence of the galactomannan and the presence of some additional element(s) of cell wall glycoprotein structure. The D76N and E130Q mutations showed some alteration in the spectrum of cell wall glycoproteins associating with DFG-5. One possibility we have considered is that the groove defined by D76 and E130 (Fig. 3) may be involved in recognizing these additional elements and allowing DFG-5 to distinguish between cell wall glycoproteins and secreted glycoproteins. Upon their release into the cell wall space, cell wall glycoproteins, having a processed N-linked galactomannan, are incorporated into the cell wall glucan/chitin matrix by GH72 family lichenin transferases (Kar et al., 2019). Secreted glycoproteins, which have a full-length N-linked galactomannan, are not recognized as substrates for the lichenin transferases and are therefore released into the growth medium. The ability of DFG-5 to discriminate between cell wall and secreted glycoproteins is thus the key event in targeting the two different types of glycoproteins to their final destinations. Further experiments will be needed to define the basis on which DFG-5 is able to discriminate between different groups of glycoproteins. The mammalian N-acetylglucosamine-1-phosphate transferase, which discriminates between glycoproteins targeted to the lysosome and those targeted for secretion, represents a similar situation in which there is clear evidence for an enzyme being able to distinguish between different groups of glycoproteins. The basis on which this is accomplished remains to be

elucidated (Coutinho et al., 2012).

While our results provide clear evidence for the role of DFG-5 in extracellular targeting of cell wall and secreted glycoproteins in *N. crassa*, additional target mechanisms must be operating in other fungal species. For example, in both *S. cerevisiae* and *C. albicans*, studies have demonstrated that GPI-anchored cell wall proteins can be attached to the cell wall through linkages between sugar residues in the GPI-anchor and cell wall β-1,6-glucan (Kapteyn et al., 1996; Kollar et al., 1997; Lu et al., 1995). This would require a mechanism different from the DFG-5-based mechanism we describe in this report. The mechanism we have defined would be incapable of using GPI anchors or β-1,6-glucan. In *S. cerevisiae*, PIR proteins have been found to be attached by a transglutaminase reaction to the cell wall β-1,3-glucan (Ecker et al., 2006). These observations indicate that additional mechanisms exist for attaching glycoproteins to fungal cell walls. We previously characterized *C. albicans* mutants deficient in CaDfg5p and CaDcwi1p and estimated that approximately 60% of the glycoprotein incorporation into the cell wall was dependent upon the presence of the Dfg5p and Dcw1p (Ao et al., 2015). However, *C. albicans* is thought to lack lichenin, suggesting that other polysaccharides might be able to be used in a DFG-5-based incorporation system. Furthermore, since only 60% of its cell wall glycoproteins seem to require the DFG-5-based incorporation system, *C. albicans* must have some additional mechanisms for the incorporation of cell wall glycoproteins. While the DFG-5-based mechanism described above is the major system operating for incorporation of cell wall glycoproteins in *N. crassa*, the research literature clearly indicates that other fungi are using additional mechanisms for the incorporation of their cell wall glycoproteins.

5. Conclusions

DFG-5 is an α-1,6-mannanase that recognizes the α-1,6-mannose backbone of the N-linked galactomannan on cell wall glycoproteins. We show that DFG-5 has an enzymatic activity. The active site is found in a groove containing D116 and D117. Two additional mutations at D76 and E130 define a second groove that is essential for DFG-5 function. The available evidence strongly suggests that the *N. crassa* enzyme digests the α-1,6-mannose backbone of N-linked galactomannan to generate a processed galactomannan structure. The processed galactomannan is subsequently used by lichenin transferases to attach cell wall glycoproteins into the cell wall matrix. DFG-5 binds cell wall glycoproteins but does not bind secreted glycoproteins. By specially recognizing cell wall glycoproteins and targeting them for cell wall incorporation, DFG-5 plays a key role in trafficking extracellular proteins to their final destinations. Cell wall glycoproteins are recognized by DFG-5 and have their galactomannans processed, which targets them for cell wall incorporation. Secreted glycoproteins are not recognized by DFG-5 and do not have their galactomannans processed, which targets them for release into the growth medium.

CRediT authorship contribution statement

Pavan K. Patel: Conceptualization, Formal analysis, Investigation, Methodology, Writing – review & editing. **Sook Keng Tung:** Investigation. **Sara Porfirio:** Investigation, Methodology. **Roberto Sonon:** Investigation, Methodology. **Parastoo Azadi:** Funding acquisition, Project administration, Supervision, Writing – review & editing. **Stephen J. Free:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors 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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Appendix A. Supplementary data

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