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G-protein coupled receptor-mediated nutrient sensing and developmental control in *Aspergillus nidulans*

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Summary

Nutrient sensing and utilisation are fundamental for all life forms. As heterotrophs, fungi have evolved a diverse range of mechanisms for sensing and taking up various nutrients. Despite its importance, only a limited number of nutrient receptors and their corresponding ligands have been identified in fungi. G-protein coupled receptors (GPCRs) are the largest family of transmembrane receptors. The *Aspergillus nidulans* genome encodes 16 putative GPCRs, but only a few have been functionally characterised. Our previous study showed the increased expression of an uncharacterised putative GPCR, *gprH*, during carbon starvation. GprH appears conserved throughout numerous filamentous fungi. Here, we reveal that GprH is a putative receptor involved in glucose and tryptophan sensing. The absence of GprH results in a reduction in cAMP levels and PKA activity upon adding glucose or tryptophan to starved cells. GprH is pre-formed in conidia and is increasingly active during carbon starvation, where it plays a role in glucose uptake and the recovery of hyphal growth. GprH also represses sexual development under conditions favouring sexual fruiting and during carbon starvation in submerged cultures. In summary, the GprH nutrient-sensing system functions upstream of the cAMP-PKA pathway, influences primary metabolism and hyphal growth, while represses sexual development in *A. nidulans*.

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Introduction

Fungi are equipped with a plethora of receptors that sense both abiotic and biotic stimuli. These receptors facilitate the coordination of the appropriate fungal development, morphogenesis and metabolism with the surrounding environment. G-protein coupled receptors (GPCRs) are the largest family of transmembrane receptors, which detect predominantly unknown extracellular signals and initiate intracellular signalling cascades (Xue *et al.*, 2008). GPCRs sense a diverse array of stimuli including light, sugars, amino acids and pheromones (Maller, 2003). All GPCRs possess seven transmembrane domains. GPCRs initiate downstream signalling through the associated heterotrimeric G-proteins. Sensitisation of a GPCR by a ligand results in the exchange of GTP for GDP on the Gα subunit, leading to its dissociation from the Gβγ subunits, and both can subsequently interact with effector proteins that regulate downstream signalling (Xue *et al.*, 2008). In fungi, GPCR-regulated signalling pathways include the cAMP-dependent protein kinase (PKA) and the mitogen-activated protein kinase (MAPK) cascades, which regulate metabolism, growth, morphogenesis, mating, stress responses and virulence (Lengeler *et al.*, 2000; Rispail *et al.*, 2009).

Heterotrimeric G-proteins are conserved in all eukaryotes and are crucial components relaying external signals into the cells. The sequential sensitisation and activation of G-proteins translate these signals into the appropriate transcriptional, biochemical and behavioural alterations. Regulators of G-protein signalling (RGSs) control the intensity and duration of the G-protein signal (Yu, 2006). *A. nidulans* represents the best characterised fungal G-protein signalling system, and includes three α subunits plus a single β and γ subunit. The activated Gα, FadA,

promotes vegetative growth and inhibits both asexual and sexual development (Yu *et al*., 1996). The RGS, FlbA, antagonises FadA and is required to control vegetative growth and permit asexual development (Yu *et al*., 1996). The Gβ, SfaD, is required for normal vegetative growth and the proper downregulation of conidiation (Rosén *et al*., 1999). SfaD and the Gγ, GpgA, are also required for sexual development (Seo *et al*., 2005). Alternatively, the Gα, GanB, in combination with SfaD, GpgA and the pathway specific antagoniser, RgsA, acts as a carbon sensing system involved in early cAMP-dependent germination and the negative regulation of asexual development (Lafon *et al*., 2005). The third Gα, GanA, remains to be characterised. Therefore, G-protein signalling represents a fundamental, and the initial, intracellular step in the conversion of extracellular signals into transcriptional, biochemical and behavioural responses, as demonstrated by the influence of the aforementioned pathways in nutrient sensing, vegetative growth, asexual and sexual development. However, the extracellular receptors for these pathways are less well understood.

The *Saccharomyces cerevisiae* genome only encodes two pheromone receptors, Ste2 and Ste3, and a glucose sensor Gpr1 (http://www.yeastgenome.org). These represent the best functionally characterised examples of GPCRs (Herskowitz, 1995; Kraakman *et al*., 1999; Pan and Heitman, 1999). However, genome sequencing projects have identified numerous putative GPCRs in filamentous fungi. Primarily, the filamentous fungal orthologues of the *S. cerevisiae* pheromone and glucose receptors have been characterised. The *A. nidulans* genome encodes 16 putative GPCRs (Lafon *et al*., 2006; Yu, 2006), but the information on the function of these receptors is limited. Similar to Ste2/3, the *A. nidulans* pheromone receptors, PreA/GprA and PreB/GprB, are required for self-fertilisation, but not vegetative growth or asexual sporulation, Hülle cell formation, or even sexual fruiting body (cleistothecium) formation in outcrosses (Seo *et al*., 2004). These GPCRs bind to the opposite mating pheromone, stimulating the G-protein subunits and the mating MAPK cascade resulting in the activation of MpkB and subsequently SteA, which are required for sexual development (Vallim *et al*., 2000). In *Neurospora crassa*, nine potential GPCRs have been identified, including the two pheromone receptors Pre-1 and Pre-2 (Galagan *et al*., 2003; Kim and Borkovich, 2004; Kim *et al*., 2012). Recently, all 15 GPCRs in aflatoxin-producing *Aspergillus flavus* were characterised again revealing two putative pheromone receptors (Affeldt *et al*., 2014).

Nutrient sensing is fundamental to fungi and subsequently has a wide-ranging impact upon numerous aspects of fungal biology. Yet despite the importance of nutrient sensing, only a limited number of receptors and their corresponding ligands have been identified in fungi. The majority of characterised, nutrient-sensing GPCRs in fungi have been shown to be involved in sugar sensing. In *S. cerevisiae*, the Gpr1 glucose receptor activates the Gα subunit, Gpa2 and the cAMP-PKA pathway, controlling trehalose mobilisation, stress resistance and ribosomal gene expression (Lorenz and Heitman, 1997; Xue *et al*., 1998; Kraakman *et al*., 1999). Similarly, the *Schizosaccharomyces pombe* Git3 receptor and the *Candida albicans* Gpr1 homologue sense glucose and activate cAMP signalling, regulating yeast-to-hyphal transition and morphogenesis (Welton and Hoffman, 2000; Miwa *et al*., 2004; Maidan *et al*., 2005a). In *N. crassa*, the Gpr1 homologue, Gpr-4, interacts with the Gα subunit, Gna-1, influencing cAMP production and asexual development in a carbon source-dependent manner (Li and Borkovich, 2006). In *A. nidulans*, a putative carbon source receptor, GprD, influences hyphal growth, germination and primary metabolism (Han *et al*., 2004; de Souza *et al*., 2013). Interestingly, the pheromone receptor GprB and the carbon source receptor GprD were found to possess opposing functions in the regulation of sexual development (Seo *et al*., 2004).

Amino acids are also an important source of nutrients for fungi. *S. cerevisiae* detects amino acids via a specialised sensor system, including the amino acid permease, Gap1, and the Ssy1–Ptr3–Ssy5 system (Bahn *et al*., 2007). In filamentous fungi, GPCRs can also sense amino acids. For example, the *C. albicans* glucose receptor Gpr1 also detects amino acids such as methionine (Miwa *et al*., 2004; Maidan *et al*., 2005b). In contrast, the *Cryptococcus neoformans* receptor Gpr4 senses methionine and activates the cAMP-PKA pathway, but is not a major receptor for glucose, regulating capsule formation and mating (Xue *et al*., 2006). Distinct from all other described nutrient-sensing GPCRs, Stm1 in *S. pombe* has been proposed to inhibit vegetative growth and cAMP production, while increasing meiosis-specific proteins and sexual development, during nitrogen limitation (Chung *et al*., 2003). However, the ligand for the Stm1 receptor is unknown.

Filamentous ascomycetes of the genus *Aspergillus* include a diverse range of fungi with biotechnological and medical significance. These *Aspergilli* include the industrial cell factory, *A. niger*, and pathogens of both humans and plants, such as *Aspergillus fumigatus* and *A. flavus*. *A. nidulans* has represented an important genetic system for the study of fungal development, metabolism and gene regulation (Goldman and Osmani, 2008). In turn, the knowledge generated through the investigation of *A. nidulans* has had a direct impact on the understanding of less amenable *Aspergilli*. The present study adopted *A. nidulans* for the investigation of GPCR-mediated nutrient sensing and its influence on fungal metabolism and development. Previously, we showed that mRNA levels of an uncharacterised GPCR, *gprH*, were increased during carbon starvation (Krohn *et al*., 2014). GprH orthologues

were identified in numerous filamentous fungi, implying the possible existence of a conserved signalling pathway. In order to further understand its potential role in sensing nutrients, we examined the functions of GprH in various aspects of fungal biology. Our present study shows a series of data leading to the conclusions that GprH is a putative receptor for glucose and tryptophan, which transduces signals, at least in part, via the cAMP-PKA pathway. In addition to influencing primary metabolism, GprH also represses sexual development during carbon starvation.

Results

Identification of gprH *as part of the carbon starvation response*

A previous genome-wide microarray investigation of the transcriptional response of *A. nidulans* to long-term carbon starvation revealed the significant differential expression ($p < 0.01$) of several GPCR encoding genes, from which *gprH* was the most highly induced, showing a 1.9 log twofold change induction (Krohn *et al*., 2014; Supporting Information Table S1; GEO Number GSE42732). This data suggested that GprH may be involved in a nutrient-sensing system that is active during carbon starvation. The *A. nidulans gprH* (AN8262) gene model (Supporting Information Fig. S1) is supported by RNA-seq data (available at http://www.aspgd.org). The organisation of the predicted GprH protein was assessed using the SMART interface (http://smart.embl-heidelberg.de/) and the Phyre2 protein structure prediction tool (Kelley and Sternberg, 2009). The GprH protein was predicted to contain seven transmembrane domains, representative of GPCRs, plus a cytoplasmic loop and tail (Supporting Information Fig. S1). In addition, three overlapping Pfam domains were identified within the same region; the Dicty_CAR cAMP receptor (PF05462, 7.8e-14), the 7tm_2 secretin receptor (PF00002, 2.2e-12) and the Git3 glucose receptor (PF11710, 1.4e-10). Phylogenetic analyses identified numerous GprH homologues within prominent filamentous fungal species (protein identity greater than 50%), including multiple *Aspergilli* plus numerous plant pathogens and saprophytic fungi (Fig. 1; Supporting Information Table S2). Therefore, GprH may represent a conserved, uncharacterised GPCR involved in nutrient signalling systems in filamentous fungi, potentially influencing fungal metabolism and development.

Subsequently, the function of GprH in nutrient sensing, carbon starvation and fungal development was assessed further via the generation of three Δ*gprH* mutants. All three transformants showed essentially the same phenotypes, and a single Δ*gprH* strain was selected for the majority of the analyses. No growth or conidiation defects were observed in the *ΔgprH* strain, while it also showed the same growth abilities as the wild-type strain on several different carbon and nitrogen sources (Supporting Information Text S1; data not shown). To ensure that the Δ*gprH* strain used throughout this study (containing *ΔgprH::argB*$^+$ deletion cassette) did not possess additional mutations, it was sexually crossed with a strain containing the non-functional *argB2* mutation, which is auxotrophic for arginine. The subsequent *ΔgprH::argB*$^+$ progeny, which were arginine prototrophs, were selected. Phenotypic investigation of the progeny revealed no significant differences to the wild-type strain, except for enhanced cleistothecia production similar to that of Δ*gprH* strain (see below). This strongly indicates that GprH is responsible for the observed phenotypes.

Genetics and RT-qPCR were used to evaluate the influence of both short- and long-term carbon starvation on the expression of, and interplay between, (i) the putative nutrient receptor, *gprH*; (ii) the putative carbon receptor and repressor of sexual development, *gprD* (de Souza *et al*., 2013 and Han *et al*., 2004); and (iii) the pheromone receptor and inducer of sexual development, *gprB* (Seo *et al*., 2004). The possible genetic interactions between two putative nutrient receptors, GprH and GprD, were evaluated by constructing a *ΔgprH ΔgprD* double mutant by genetic mediated transformation. The wild-type, the pre-existing Δ*gprD* (Han *et al*., 2004), the Δ*gprH* and the *ΔgprH ΔgprD* strains were grown in minimal media plus 1% glucose then subsequently washed with, and transferred to, minimal media lacking a carbon source for 0, 1, 2, 4 and 8 h (short-term starvation), and 24, 120 and 192 h (long-term starvation). In the wild-type strain, *gprH* showed a significant level of induction throughout short- and long-term carbon starvation, while *gprD* only showed a moderate level of induction (Fig. 2; Supporting Information Fig. S2). In the Δ*gprD* strain, the expression of the alternative putative nutrient receptor, *gprH*, showed a moderate reduction in the transcriptional response to carbon starvation when compared with the wild-type strain, suggesting that these receptors may function in parallel or are interacting. In contrast, in the Δ*gprH* strain, the expression of *gprD* was unaffected by the absence of GprH. Short-term carbon starvation significantly induced *gprB* expression in the Δ*gprH* and Δ*gprD* strains, a phenotype that was not observed in the wild-type or *ΔgprH ΔgprD* strain, again showing a potential interaction between these three GPCRs. Post-long-term starvation, *gprB* was induced in all strains, yet to a higher level in the wild-type and Δ*gprD* strains.

Therefore, the putative nutrient receptor GprH was induced during carbon starvation, and this induction was maintained throughout short- and long-term starvation, which was in contrast to the other characterised putative carbon receptor GprD. The absence of either nutrient receptor resulted in the increased expression of the phero-

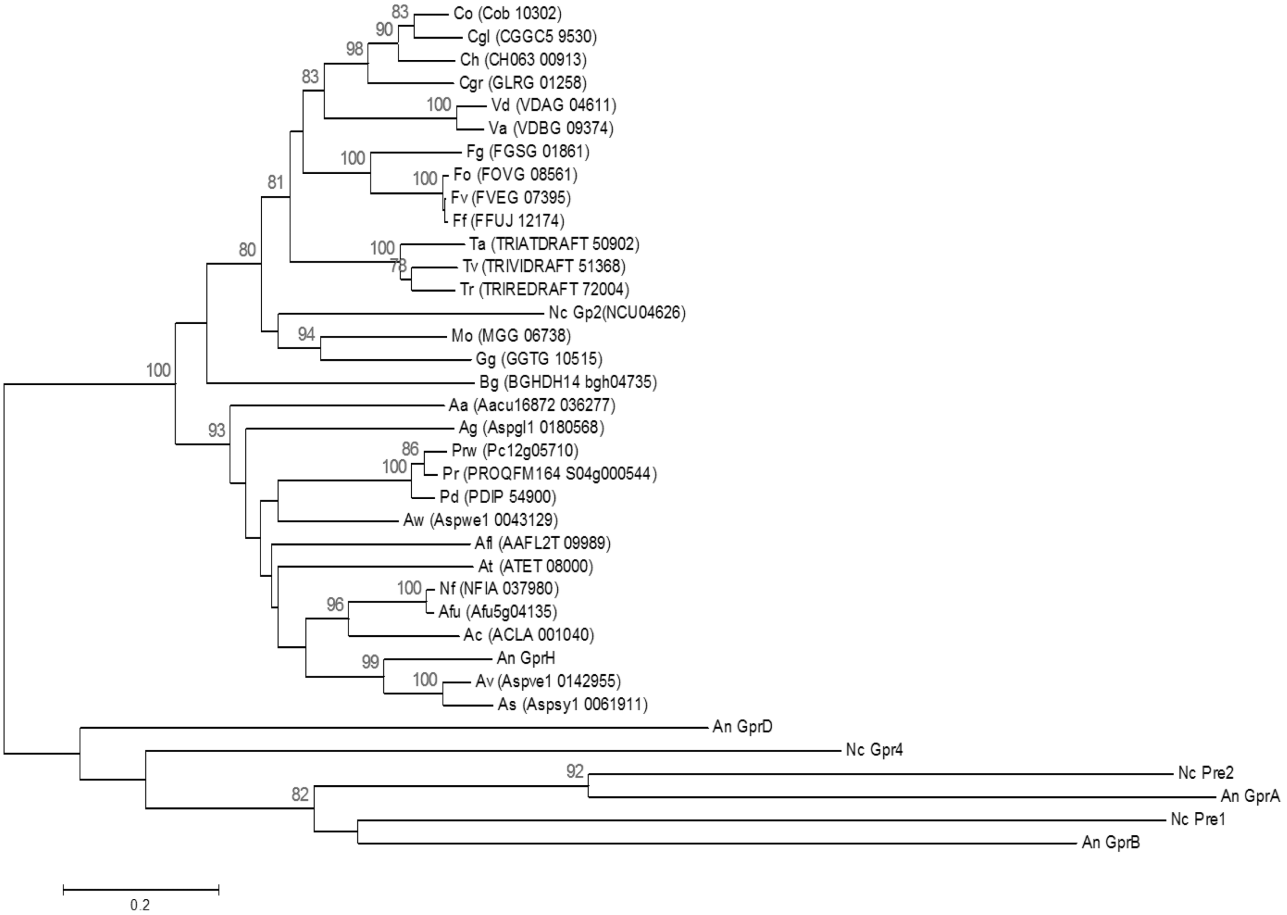

Fig. 1. Conservation of GprH homologues in filamentous fungi. Phylogeny based on the amino acid sequences of the filamentous fungal GprH homologues (protein identity greater than 1e-45). Bootstrap values greater than 70% are presented (500 replicates). Branch length is equivalent to evolutionary distance, as calculated using the Poisson correction method, and represents the number of amino acid substitutions per site. The outgroup consists of characterised filamentous fungal GPCRs, including the putative pheromone and sugar receptors in *A. nidulans* and *N. crassa*. For a full list of abbreviated species names and their homology to GprH (E-values), refer to Supporting Information Table S1. An, *Aspergillus nidulans*; Av, *Aspergillus versicolor*; As, *Aspergillus sydowii*; Aw, *Aspergillus wentii*; Ac, *Aspergillus clavatus*; Afu, *Aspergillus fumigatus*; At, *Aspergillus terreus*; Aa, *Aspergillus aculeatus*; Afl, *Aspergillus flavus*; Ag, *Aspergillus glaucus*; Nf, *Neosartorya fischeri*; Pr, *Penicillium roqueforti*; Pc, *Penicillium chrysogenum Wisconsin*; Pd, *Penicillium digitatum*; Co, *Colletotrichum orbiculare*; Cgl, *Colletotrichum gloeosporioides*; Fo, *Fusarium oxysporum* f. sp. *cubense*; Ch, *Colletotrichum higginsianum*; Fv, *Fusarium verticillioides*; Ff, *Fusarium fujikuroi*; Vd, *Verticillium dahliae*; Va, *Verticillium alfalfae*; Tr, *Trichoderma reesei*; Fg, *Fusarium graminearum*; Cgr, *Colletotrichum graminicola*; Ta, *Trichoderma atroviride*; Gg, *Gaeumannomyces graminis* var. *tritici*; Mo, *Magnaporthe oryzae*; Tv, *Trichoderma virens*; Nc, *Neurospora crassa*; Bg, *Blumeria graminis* f. sp. *hordei*.

mone receptor and positive regulator of sexual development during carbon starvation, *gprB*. Subsequently, the role of GprH in regulating fungal metabolism and development during carbon starvation was investigated.

GprH influences the transcriptional regulation of primary metabolism during carbon starvation

Primarily, genome-wide microarray analyses were utilised to further investigate the function of GprH during carbon starvation. Due to the fact that this investigation focused on a putative nutrient-sensing system, which potentially partakes in a rapid response to environmental change, short-term carbon starvation was utilised, which was in contrast to the published prolonged carbon starvation microarray analysis that identified *gprH* as being induced by starvation (Krohn *et al.*, 2014), but in accordance with the RT-qPCR analysis that showed GprH influenced the expression of *gprB* after just a few hours starvation (Fig. 2). The wild-type and one of the Δ*gprH* strains were grown in minimal media plus 1% glucose for 24 h and subsequently washed with, and transferred to, minimal media lacking a carbon source for 4 and 8 h. The genes transcriptionally modulated in the two strains post-carbon starvation were identified. Both strains demonstrated a large transcriptional response to carbon starvation, upregulating 1330/1326 and downregulating 1520/1683 genes in the wild-type and Δ*gprH* strains respectively. Despite a significant proportion of these tran-

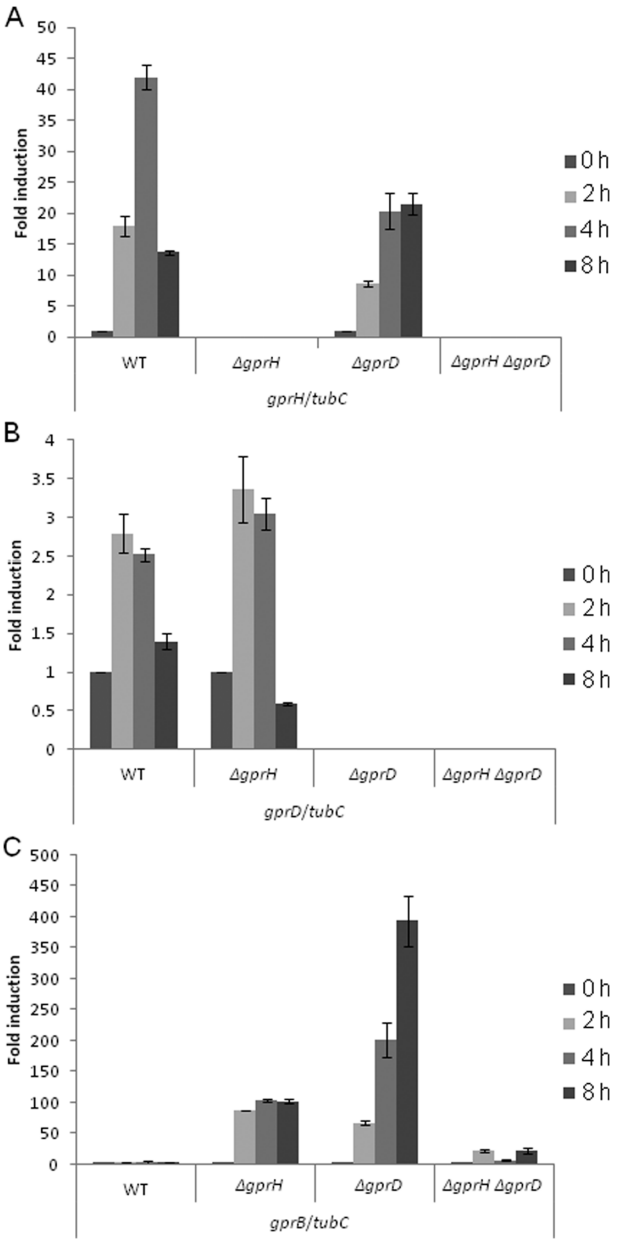

Fig. 2. Carbon starvation induced expression of GprH. The wild-type, Δ*gprH*, Δ*gprD* and the Δ*gprH* Δ*gprD* strains were grown in minimal media containing 1% glucose (0 h) and then transferred to minimal media containing no carbon for 2, 4 and 8 h. The abundance of the respective mRNAs was normalised using *tubC*. Presented is the fold change in normalised mRNA abundance post-transfer from glucose to the respective carbon starvation condition.
A. Fold induction of *gprH*.
B. Fold induction of *gprD*.
C. Fold induction of *gprB*.

scriptional responses being shared by the two strains, approximately half the modulations in gene expression were strain specific (Fig. 3).

The analysis of the overrepresented gene ontologies (GO terms), within the transcriptionally modulated genes, generated a profile for both strains. Subsequently, the strain-specific modulations in the transcriptional profile were identified. Both strains demonstrated an upregulation in autophagy, fatty acid β-oxidation and amino acid catabolism, while a downregulation in glycolysis, mitochondrial components and respiration (Table 1). The wild-type strain-specific transcriptional alterations included the upregulation of additional autophagy-related processes, while the downregulation of amino acid biosynthesis, the TCA cycle and oxidoreductase activity (Table 2). The Δ*gprH* strains showed a strain-specific transcriptional upregulation in TOR signalling, cellular response to chemical stimulus and hydrolase activity, while a downregulation in lysine/methionine biosynthesis and protein translation (Table 3). Therefore, GprH impacts upon the carbon starvation response through its influence on autophagy, primary carbon and amino acid metabolism, and mitochondrial function. The influence of GprH on these metabolic processes may subsequently be regulated via the antagonistic actions of the PKA and TOR pathways, known to control growth and metabolism in response to nutrient signals.

GprH influences primary metabolism during glucose metabolism and carbon starvation

The ^{1}H NMR analyses were utilised to investigate the influence of GprH on the metabolome during glucose metabolism and post-6 or 12 h carbon starvation, slightly longer than the microarray analysis to enable the observation of the potential impact of such transcriptional modulations on the metabolic profile. Principal component analysis (PCA) analysis was used to determine the main contributions to variation in the metabolite profile data. As highlighted in Fig. 4, carbon starvation is the dominant source of variation underlying the principal component 1, contributing to 53% of the variation, whereas the strains were the main basis for principal component 2 separation (22% of explained variation). To further investigate the relative contributions of the strains and carbon starvation, an ANOVA analysis followed by a Tukey's test was performed for all 33 metabolites measured in all samples (Supporting Information Table S3). Both the wild-type and the Δ*gprH* strains demonstrated a profile, where the sugars and sugar alcohols steadily decreased during carbon starvation. Free lipid compounds, such as choline and sn-glycero-3-phosphocholine, and an unknown polysaccharide increased during starvation, potentially representative of the breakdown of fungal cell membranes and cell wall. The levels of free amino acids oscillated but generally increased due to their increased liberation by proteases. The similarity in the metabolite profile between both strains during carbon starvation was in accordance to the substantial similarity previously observed in the

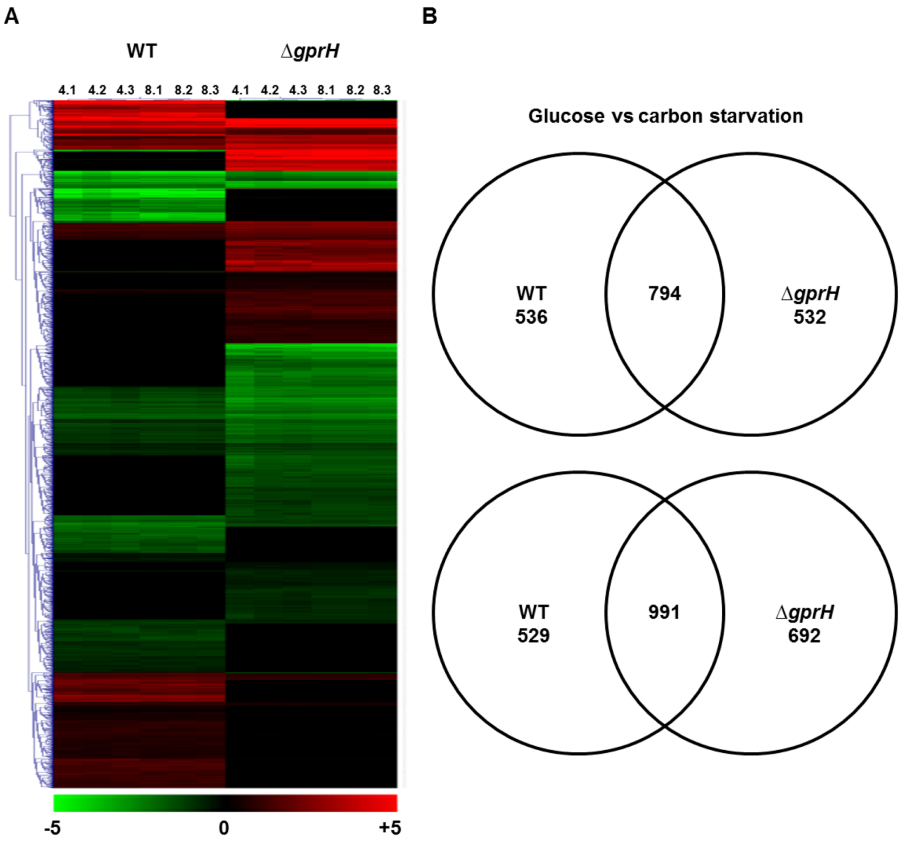

Fig. 3. The transcriptome of the wild-type and Δ*gprH* strains post-carbon starvation.
A. The HCL analysis of the genes transcriptionally modulated post-transfer from glucose containing media to 4 and 8 h starvation in wild-type and Δ*gprH* strains.
B. Venn diagram of the genes transcriptionally modulated post-starvation in wild-type and Δ*gprH* strains reveals the strain-specific alterations.

transcriptional profile. Despite both strains demonstrating a similar response to carbon starvation, a comparative analysis of the concentration of the identified compounds revealed subtle differences that were used to elucidate putative functions of GprH (Fig. 4; Supporting Information Table S3). During growth on glucose (0 h), the Δ*gprH* strain possessed a higher level of sugars and energy-containing molecules, especially acetate, in addition to a higher level of free amino acid, such as glutamine, and the nucleoside uridine. Post-carbon starvation, the Δ*gprH* strain maintained a higher level of sugars, such as glucose and fructose, while also showing a lower level of tyrosine and uridine when compared with the wild-type strain. Therefore, GprH appeared to influence both primary carbon and amino acid metabolism during glucose sustained growth and during carbon starvation.

GprH is involved in glucose and tryptophan sensing, in turn inducing cAMP production and PKA activity

The previous transcriptomic and metabolomic studies implied that *gprH* was induced by carbon starvation and influenced fungal primary metabolism. Subsequently, to investigate the involvement of GprH in both carbon and nitrogen nutrient-sensing systems, the ability of the wild-type and Δ*gprH* strains to induce a burst of cAMP synthesis and PKA activity in response to glucose and amino acids was assessed. The two strains were cultured in complete media (YG) for 16 h, then washed and incubated in media containing no glucose or nitrogen for 4 h, prior to the addition of the amino acid groups or glucose. Thirteen amino acids were divided into three groups: (i) leucine, isoleucine, histidine and valine; (ii) lysine, methionine and tryptophan; (iii) alanine, phenylalanine and tyrosine; and (iv) arginine, glutamic acid and threonine. The wild-type strain demonstrated an increase in cAMP during starvation and subsequently a burst of cAMP production post the addition of all amino acid groups and glucose (Fig. 5). The Δ*gprH* strain started with a higher level of cAMP compared with the wild-type strain in complete media and did not accumulate cAMP during starvation (Fig. 5). In addition, the Δ*gprH* strain showed no burst of cAMP in response to amino acid group B or glucose (Fig. 5). Subsequently, the amino acids of group B were independently assessed, revealing that Δ*gprH* strain was unable to induce a burst of cAMP in response to detecting tryptophan (Fig. 6A). Therefore, GprH appears to play a role in both glucose and tryptophan signalling mechanisms in *A. nidulans*.

The function of GprH as a putative receptor involved in both glucose and tryptophan signalling, which acts as an upstream regulator of the cAMP-PKA pathway, was confirmed using the same experimental design as the cAMP

Table 1. Overrepresented GO terms transcriptionally modulated during carbon starvation in both the wild-type and Δ*gprH* strains.

GO term	Description	*p* Value	Reg.	GO class
0006878	Cellular copper ion homeostasis	0.000296	↗	BP
0043687	Post-translational protein modification	8.97E-06	↗	BP
0000045	Autophagic vacuole assembly	3.86E-05	↗	BP
0033539	Fatty acid beta-oxidation using acyl-CoA dehydrogenase	0.000296	↗	BP
0009083	Branched chain family amino acid catabolic process	0.000111	↗	BP
0006122	Mitochondrial electron transport, ubiquinol to cytochrome c	0.005052	↘	BP
0032543	Mitochondrial translation	0.001104	↘	BP
0033108	Mitochondrial respiratory chain complex assembly	0.00065	↘	BP
0006783	Heme biosynthetic process	0.00065	↘	BP
0000105	Histidine biosynthetic process	0.005052	↘	BP
0006096	Glycolysis	0.002925	↘	BP
0006744	Ubiquinone biosynthetic process	0.000348	↘	BP
0016226	Iron-sulfur cluster assembly	2.88E-07	↘	BP
0007007	Inner mitochondrial membrane organisation	0.000188	↘	BP
0009102	Biotin biosynthetic process	0.001715	↘	BP
0030150	Protein import into mitochondrial matrix	1.17E-06	↘	BP
0005763	Mitochondrial small ribosomal subunit	6.67E-08	↘	CC
0005742	Mitochondrial outer membrane translocase complex	0.000348	↘	CC
0005750	Mitochondrial respiratory chain complex III	0.005052	↘	CC
0005758	Mitochondrial intermembrane space	7.41E-07	↘	CC
0031307	Integral to mitochondrial outer membrane	0.00135	↘	CC
0005744	Mitochondrial inner membrane pre-sequence translocase complex	0.00135	↘	CC
0045254	Pyruvate dehydrogenase complex	0.001715	↘	CC
0005732	Small nucleolar ribonucleoprotein complex	0.007755	↘	CC
0005762	Mitochondrial large ribosomal subunit	8.55E-12	↘	CC
0008137	NADH dehydrogenase (ubiquinone) activity	0.000264	↘	MF
0003735	Structural constituent of ribosome	1.45E-10	↘	MF
0008121	Ubiquinol-cytochrome-c reductase activity	0.00135	↘	MF
0015266	Protein channel activity	8.75E-05	↘	MF

investigation. However, this study assessed PKA activity in the wild-type and Δ*gprH* strains (Fig. 6B). In the wild-type strain, PKA activity was constantly higher than the Δ*gprH* strain post the addition of glucose or tryptophan to the carbon and nitrogen starved hyphae. Therefore, GprH appears to be involved in both the glucose and tryptophan sensing mechanisms, while functioning upstream of the cAMP-PKA pathway.

Table 2. Wild-type-specific overrepresented GO terms transcriptionally modulated during carbon starvation.

GO term	Description	*p* Value	Reg.	GO class
0009896	Positive regulation of catabolic process	0.001324	↗	BP
0000422	Mitochondrion degradation	0.00129	↗	BP
0050794	Regulation of cellular process	0.000607	↗	BP
0032258	CVT pathway	5.93E-05	↗	BP
0032446	Protein modification by small protein conjugation	0.000646	↗	BP
0006366	Transcription from RNA polymerase II promoter	0.001239	↗	BP
0034727	Piecemeal microautophagy of nucleus	9.97E-06	↗	BP
0000715	Nucleotide-excision repair, DNA damage recognition	0.001324	↗	BP
0043189	H4/H2A histone acetyltransferase complex	0.001533	↗	CC
0000407	Pre-autophagosomal structure	0.000659	↗	CC
0044437	Vacuolar part	0.001481	↗	CC
0009082	Branched chain family amino acid biosynthetic process	0.004752	↘	BP
0046165	Alcohol biosynthetic process	0.003584	↘	BP
0032048	Cardiolipin metabolic process	0.001715	↘	BP
0006099	Tricarboxylic acid (TCA) cycle	0.004581	↘	BP
0005786	Signal recognition particle, endoplasmic reticulum targeting	0.001715	↘	CC
0031305	Integral to mitochondrial inner membrane	0.007181	↘	CC
0031314	Extrinsic to mitochondrial inner membrane	0.005052	↘	CC
0016653	Oxidoreductase activity, acting on NADH or NADPH, heme protein as acceptor	0.001715	↘	MF
0005507	Copper ion binding	0.001735	↘	MF

Table 3. Δ*gprH*-specific overrepresented GO terms transcriptionally modulated during carbon starvation.

GO term	Description	*p* Value	Reg.	GO class
0031929	TOR signalling cascade	1.35E-05	↗	BP
0019521	D-gluconate metabolic process	0.000727	↗	BP
0070887	Cellular response to chemical stimulus	1.25E-06	↗	BP
0031932	TORC2 complex	0.001965	↗	CC
0071944	Cell periphery	0.001435	↗	CC
0017086	3-methyl-2-oxobutanoate dehydrogenase (lipoamide) complex	0.001965	↗	CC
0005886	Plasma membrane	0.000587	↗	CC
0016787	Hydrolase activity	0.000171	↗	MF
0006515	Misfolded or incompletely synthesised protein catabolic process	0.005916	↘	BP
0019878	Lysine biosynthetic process via aminoadipic acid	0.004804	↘	BP
0017004	Cytochrome complex assembly	0.004804	↘	BP
0033617	Mitochondrial respiratory chain complex IV assembly	0.005584	↘	BP
0006555	Methionine metabolic process	0.000121	↘	BP
0008610	Lipid biosynthetic process	0.004419	↘	BP
0006418	tRNA aminoacylation for protein translation	9.11E-08	↘	BP
0006413	Translational initiation	0.001919	↘	BP
0000466	Maturation of 5.8S rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA)	0.00221	↘	BP
0031120	snRNA pseudouridine synthesis	0.005916	↘	BP
0000055	Ribosomal large subunit export from nucleus	0.000187	↘	BP
0009152	Purine ribonucleotide biosynthetic process	0.00598	↘	BP
0033615	Mitochondrial proton-transporting ATP synthase complex assembly	0.001262	↘	BP
0045039	Protein import into mitochondrial inner membrane	0.001798	↘	BP
0000154	rRNA modification	0.001861	↘	BP
0000463	Maturation of LSU-rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA)	0.006045	↘	BP
0006544	Glycine metabolic process	0.005916	↘	BP
0044452	Nucleolar part	0.000835	↘	CC
0030687	Preribosome, large subunit precursor	5.58E-06	↘	CC
0032040	Small-subunit processome	0.010234	↘	CC
0043614	Multi-eIF complex	0.005584	↘	CC
0042719	Mitochondrial intermembrane space protein transporter complex	0.004804	↘	CC
0030686	90S preribosome	0.000571	↘	CC
0005832	Chaperonin-containing T-complex	0.000157	↘	CC
0001405	Presequence translocase-associated import motor	0.001262	↘	CC
0004812	Aminoacyl-tRNA ligase activity	1.09E-08	↘	MF
0051082	Unfolded protein binding	0.000235	↘	MF
0046912	Transferase activity, transferring acyl groups, acyl groups converted into alkyl on transfer	0.000536	↘	MF
0030515	snoRNA binding	0.000588	↘	MF

GprH influences glucose uptake and growth on glucose post-carbon starvation

The cAMP-PKA nutrient-sensing pathway is well characterised and performs an essential role in the regulation of glucose uptake and growth in fungi (Pan and Heitman, 1999; Lafon *et al*., 2005). Therefore, the influence of GprH, which appears to function upstream of the cAMP-PKA pathway, on these traits was assessed in *A. nidulans*. Previous studies have described protocols for determining glucose uptake in *A. nidulans*, which require the use of conidia and not fungal mycelia. These studies have shown that glucose uptake in germinating *A. nidulans* conidia, incubated in media containing 1.0% glucose, is an energy-dependent process (MacCabe *et al*., 2003). Subsequently, the role of GprH in glucose uptake in germinating conidia was assessed accordingly. The Δ*gprH* strain demonstrated a moderately higher capacity and lower affinity for glucose uptake than the wild-type strain (WT: Vmax = 2.08 ± 0.05 μmol of glucose h^{-1} per 2.5×10^7 conidia, Km = 10.70 ± 0.89; Δ*gprH*: Vmax = 2.82 ± 0.12 μmol of glucose h^{-1} per 2.5×10^7 conidia, Km = 11.94 ± 1.67) (Fig. 7A). Subsequently, GprH is possibly involved in glucose sensing and appears to influence the regulation of the low-affinity glucose uptake system in *A. nidulans* during conidial germination.

As previously described, the Δ*gprH* strain demonstrated wild-type growth on solid minimal and complete media, and there were no differences in the kinetics of germling formation on glucose in both the wild-type and Δ*gprH* strains (data not shown). Therefore, the influence of GprH on the recovery of growth after carbon starvation, in germlings, was investigated. An experimental design comparable with those utilised in the cAMP and PKA experiments was adopted. The Δ*gprH* germlings showed reduced growth after adding glucose to starved germlings (Fig. 7B). These results emphasised the

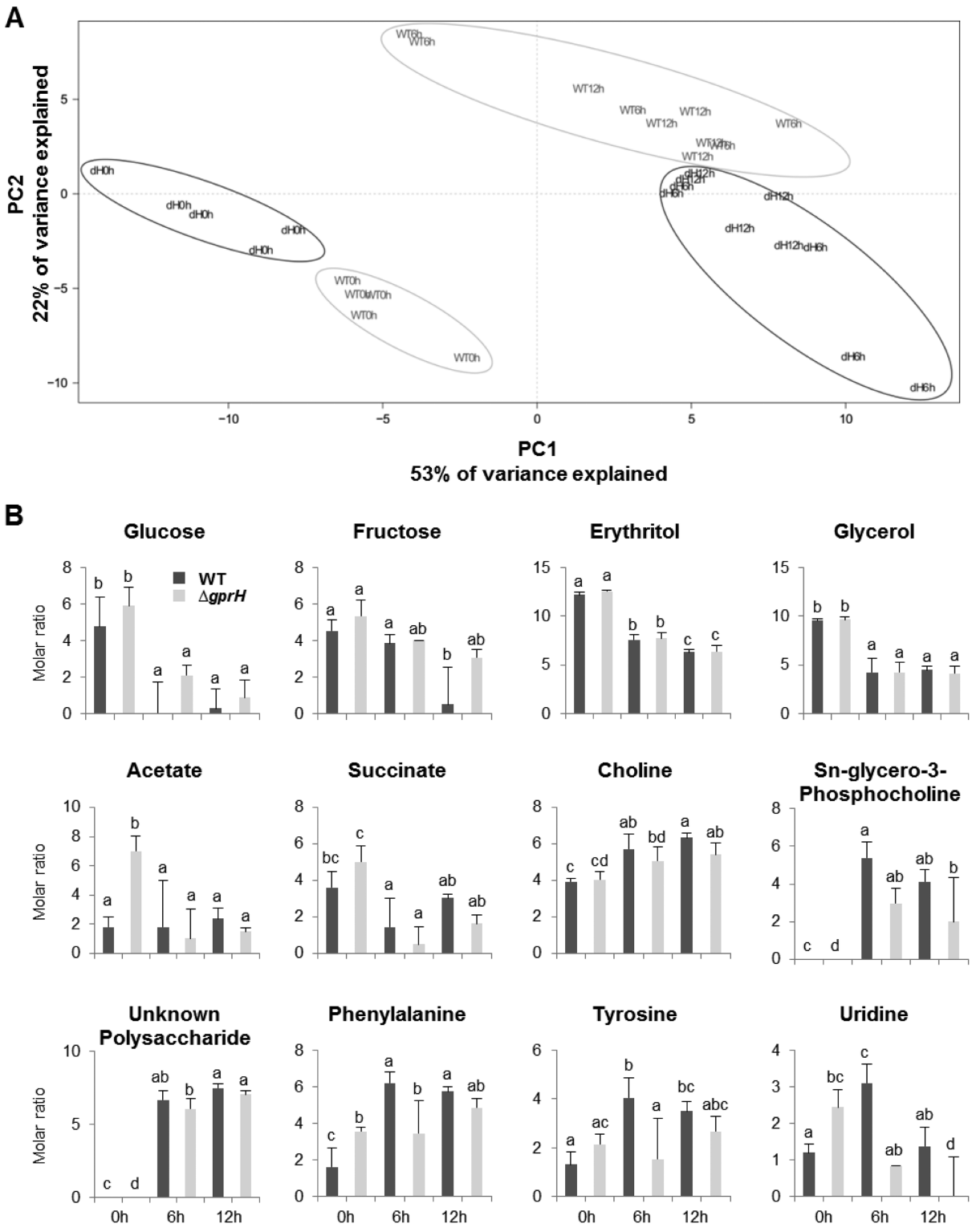

Fig. 4. The metabolome of *A. nidulans* wild-type and Δ*gprH* strains grown on glucose and post-carbon starvation.
A. The PCA plot illustrates the variance of the integrals from NMR spectral data collected at 0, 6 and 12 h post-carbon starvation. Carbon starvation is represented by PCA1 and explains 53% of the variance. Fungal strain is represented by PCA2 and explains 22% of the variance. Post-carbon starvation the metabolome of the Δ*gprH* strain was increasingly distinct from the wild-type strain.
B. The contribution of individual metabolites to the observed variance. An ANOVA analysis ($p < 0.05$) and a Tukey's test were performed for all 33 identified metabolites (Supporting Information Table S2). Presented are histograms of the molar ratios of significant metabolites in the two strains during growth on glucose or under carbon starvation.

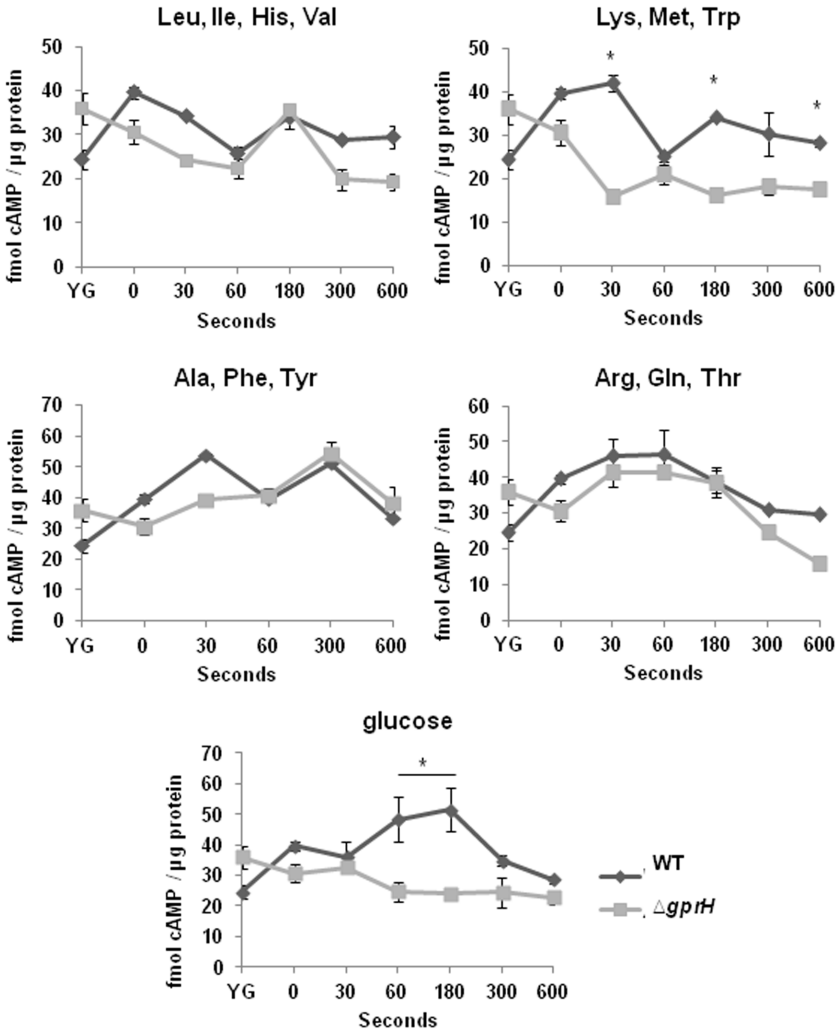

Fig. 5. GprH is a putative receptor of amino acids and glucose functioning upstream of the cAMP pathway. The *A. nidulans* wild-type and Δ*gprH* strains were cultured in complete media (YG) for 16 h, then washed and incubated in media containing no glucose for 4 h (0), prior to the addition of pools of amino acids or glucose for up to 600 s. The wild-type strain demonstrated an increase in cAMP during starvation and subsequently a burst of cAMP production post the addition of group B of the amino acids (Lys, Met and Trp) or glucose. The Δ*gprH* strain showed no burst of cAMP in response to group B of the amino acids or glucose. The results are the means of three biological repetitions, and the asterisk denotes p value < 0.01.

importance of GprH as a putative glucose sensor under carbon starvation inducing conditions.

Therefore, the putative nutrient receptor, GprH, is induced by carbon starvation where it appears to function upstream of the cAMP-PKA pathway, influencing both glucose and tryptophan nutrient-sensing mechanisms. In accordance with the function of the cAMP-PKA pathway, the absence of GprH impacted on primary carbon and amino acid metabolism, glucose uptake and fungal growth.

GprH represses sexual development and is developmentally regulated at a transcriptional level

In *A. nidulans*, sexual development requires well-nourished conditions for abundant cleistothecial production and is repressed by nutrient limitation and submerged liquid cultures (Han *et al.*, 2004). The previously characterised putative carbon sensor, GprD, also acts as a repressor of sexual development in *A. nidulans* (Han *et al.*, 2004). The absence of either GprH or GprD resulted in enhanced transcription of the pheromone receptor and sexual development activator, GprB. Therefore, the influence of GprH, a putative carbon and nitrogen sensor, on sexual development was assessed. Similar to the *ΔgprD* strain, under conditions inductive of sexual development (air-exposed then sealed nutrient-rich plates), the *ΔgprH* mutant showed a dramatic increase in the production and size of cleistothecia (Fig. 8A). The same was observed for the two other independent *ΔgprH* transformants (data not shown).

Subsequently, the levels of *gprH* mRNA during the life cycle and sexual development of *A. nidulans* were examined by Northern blot analysis (Fig. 8B). Within the conidia and ascospores, *gprH* accumulated to the highest level. During the hyphal growth in submerged nutrient-rich media, where neither conidia nor ascospores are formed, *gprH* accumulation was initially very low at 6 h, while post-12 h *gprH* accumulation peaked prior to returning to a basal level. During asexual development (air exposed),

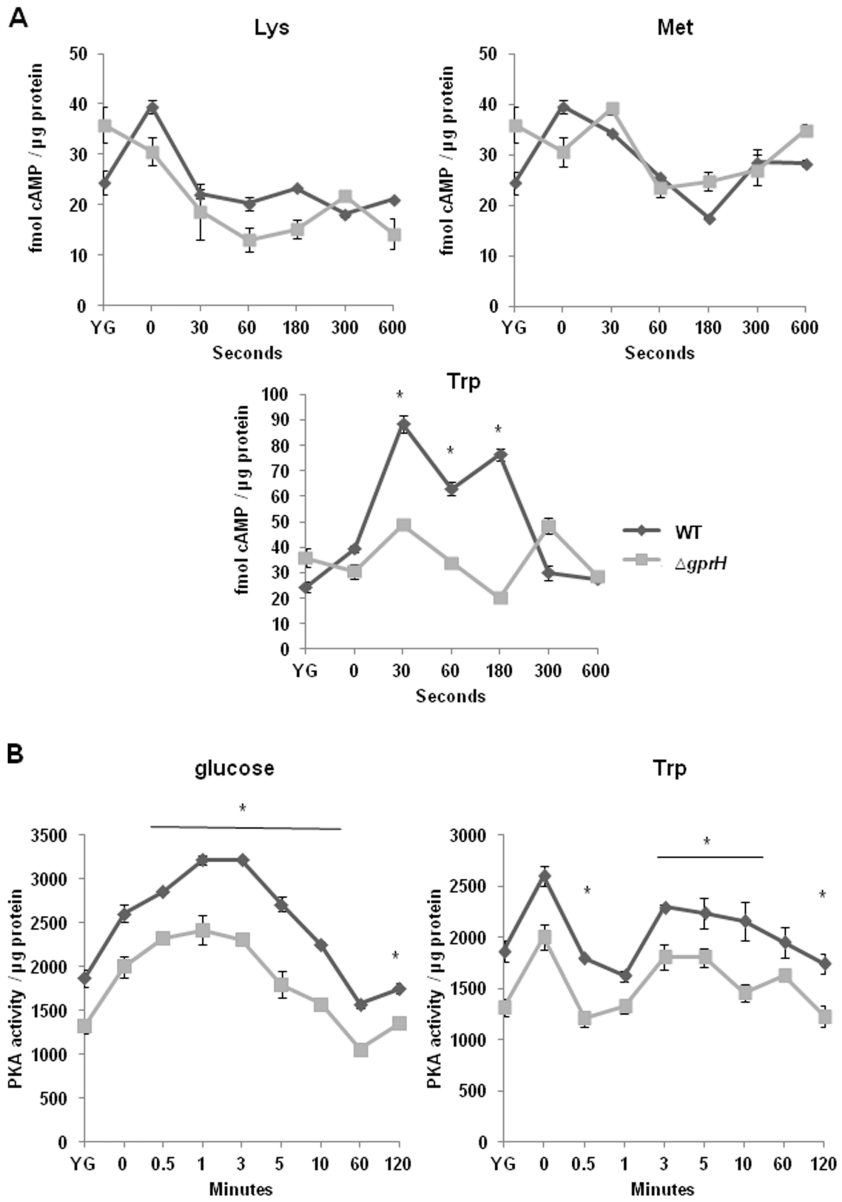

Fig. 6. GprH is a putative receptor of glucose and tryptophan functioning upstream of the cAMP-PKA pathway.
A. The *A. nidulans* wild-type and Δ*gprH* strains were cultured in complete media (YG) for 16 h, then washed and incubated in media containing no glucose for 4 h (0), prior to the addition of pools of individual amino acids for up to 600 s. The wild-type and not the Δ*gprH* showed an increase in cAMP during starvation and a burst of cAMP production post the addition of tryptophan.
B. The wild-type strain demonstrated higher protein kinase A (PKA) activity than the Δ*gprH* strain post-starvation and the subsequent addition of glucose or tryptophan for up to 120 min. The results are the means of three biological repetitions, and the asterisk denotes p value < 0.01.

gprH accumulation was high, peaking at 48 h when conidia were abundant, while during sexual development (air exposed + sealed), *gprH* accumulation was low, implicating that GprH may repress sexual development during these alternative developmental programmes.

Repression of sexual development during carbon starvation

During prolonged carbon, but not nitrogen, starvation in submerged cultures (liquid media), the three Δ*gprH* strains produced an abundance of cleistothecia, a phenotype that was not seen in the wild-type, Δ*gprD* and Δ*gprH*Δ*gprD* strains (Fig. 9; Supporting Information Fig. S3). This data suggests that GprH negatively regulated sexual development depending on the nutritional state of the environment, such as during prolonged carbon starvation, when the activity of GprD may diminish.

Subsequently, the expression of several transcription factors known to be involved in regulating sexual development in *A. nidulans* was evaluated by RT-qPCR during short- (2–8 h) and long-term (24–192 h) carbon starvation in submerged cultures of the wild-type, Δ*gprD*, Δ*gprH* and the Δ*gprH* Δ*gprD* strains. NsdD is a GATA-type zinc-finger transcription factor required for sexual development, and *nsdD* overexpression results in sexual development in liquid cultures (Han *et al.*, 2001). RosA is a Zn(II)2Cys6 transcription factor and a negative regulator of sexual development during carbon depletion, while the Δ*rosA* strain showed the derepression of sexual development in

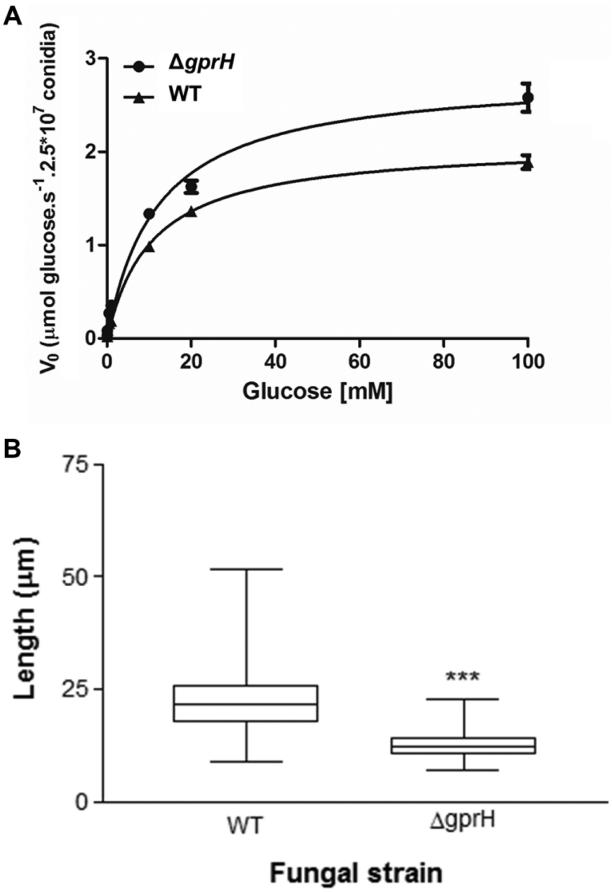

Fig. 7. GprH contributes to glucose uptake and germling growth post-carbon starvation.
A. Uptake rates for [^{14}C] glucose in germinating conidia of the *A. nidulans* wild-type and Δ*gprH* strains were determined at the indicated substrate concentrations at pH 7.0. Michaelis–Menten plots of the same data are shown (n = 3, ± , standard deviation). The Δ*gprH* strain demonstrated a moderately higher capacity and lower affinity for glucose uptake than the wild-type strain (WT: Vmax = 2.08 ± 0.05 μmol of glucose h^{-1} per 2.5×10^7 conidia, Km = 10.70 ± 0.89; Δ*gprH*: Vmax = 2.82 ± 0.12 μmol of glucose h^{-1} per 2.5×10^7 conidia, Km = 11.94 ± 1.67).
B. The *A. nidulans* wild-type and Δ*gprH* strains were cultured in minimal media for 4 h, then washed and incubated in media containing no glucose for 4 h, prior to the addition of glucose for 1 h. The length (μm) of greater than 60 germlings was measured. Three asterisks (***) denote the statistical difference of the Δ*gprH* strain compared with the wild-type ($p < 0.001$) as determined by the Mann–Whitney test.

submerge cultures (Vienken *et al.*, 2005). NosA is a paralogue of RosA but is involved in the regulation of sexual development, while the Δ*nosA* strain produces immature cleistothecia and reduced numbers of ascospores (Vienken and Fischer, 2006). During short-term carbon starvation, *nsdD* expression was comparable for all strains at 0, 2 and 4 h, but higher in the Δ*gprH* strain at 8 h, while the Δ*gprD*, Δ*gprH* and the Δ*gprH* Δ*gprD* strains all showed a dramatic reduction in *nosA* and *rosA* expression (Fig. 10). Post-long-term carbon starvation, the Δ*gprH and* Δ*gprD* strains both showed a reduction in *rosA* and *nosA* induction, while *nsdD* induction was similar to the wild-type strain. However, the Δ*gprH* Δ*gprD* strain showed increased *nsdD* induction and recovered a moderate level *rosA* induction (Supporting Information Fig. S4).

The previously characterised putative carbon receptor and sexual repressor, GprD, was proposed to impede sexual development during nutrient abundance (Han *et al.*, 2004). Here, GprH was described to be involved in glucose and tryptophan sensing, in addition to acting as a repressor of sexual development during carbon starvation. Subsequently, the absence of both GPCRs in the double Δ*gprH* Δ*gprD* strain was shown to produce no cleistothecia during carbon starvation in submerged cultures. This implied that the two putative nutrient receptors, GprH and GprD, interact during carbon starvation to regulate sexual development. Therefore, during carbon starvation, GprH may repress sexual development via modulating the expression of transcription factors involved in sexual development, in particular the repressor RosA.

Discussion

Nutrient-sensing pathways are highly conserved throughout fungi and are vital for the co-ordination of fungal developmental programmes and metabolism with the occupied environment (Lengeler *et al.*, 2000; Rispail *et al.*, 2009). In contrast to *S. cerevisiae*, filamentous fungal genomes encode numerous putative GPCRs, for which the functions and respective ligands are predominantly unknown. The *S. cerevisiae* Gpr1 glucose/sucrose receptor and cAMP-PKA pathway are the best characterised nutrient-sensing system in fungi (Xue *et al.*, 1998; Yun *et al.*, 1998; Lemaire *et al.*, 2004). Subsequently, Gpr1 homologues have been assessed in multiple fungi, where in some cases function appears conserved, while in others, deviations exist. For example, in *N. crassa*, Gpr-4 was identified as a putative carbon sensor activating the cAMP pathway (Li and Borkovich, 2006), while in *C. albicans*, Ras signalling and not the homologous Gpr1 pathway has been implicated as being responsible for glucose sensing, while Gpr1 also senses methionine (Maidan *et al.*, 2005a,b). Similarly, in the *S. pombe*, methionine also induces sexual development via a cAMP/Ste11 pathway (Schweingruber *et al.*, 1998), while in *C. neoformans*, methionine also stimulates mating filaments in a Gpr4-dependent manner, where the addition of glucose and methionine had an additive effect on the activation of cAMP pathway (Xue *et al.*, 2006). Therefore, the presented investigation of the model filamentous fungus *A. nidulans* demonstrated that GprH, the homologue of Gpr4 in *C. neoformans*, is a putative receptor of glucose and tryptophan. These two examples of GPCRs detecting glucose and amino acids in different fungal systems may represent a

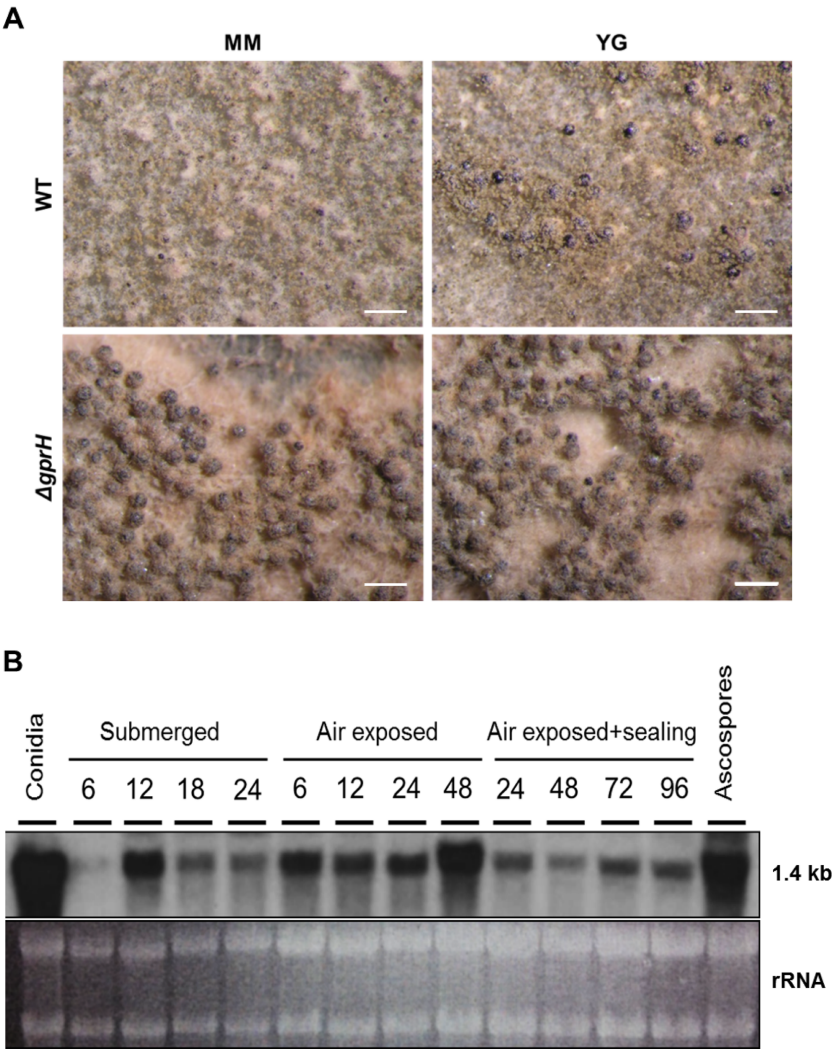

Fig. 8. GprH is developmentally regulated and important for sexual repression.
A. *Aspergillus nidulans* wild-type and Δ*gprH* strains were grown on minimal media (MM) or complete media (YG), under conditions inducing sexual development (air-exposed + sealed) for 11 days. Under both nutritional conditions, the Δ*gprH* strain demonstrated a dramatic increase in cleistothecia formation. Cleistothecia of Δ*gprH* strain were larger in size. Bar = 1 mm.
B. The accumulation of *gprH* mRNA throughout the lifecycle of *A. nidulans*. The highest level of *gprH* mRNA accumulation was observed in conidia and ascospores (sexual spores). Equal loading of total RNA was evaluated by rRNA bands.

conserved concomitant nutrient detection system, a concept that is supported by the identification of GprH homologues in numerous filamentous fungal organisms.

The interaction of GprH with intracellular G-proteins remains to be determined, as is the case for the other putative carbon sensor and sexual repressor, GprD. However, the roles of two of the three Gα subunits, FadA and GanB, are known (Yu *et al*., 1996). FadA is proposed to associate with an unknown GPCR which detects growth signals and with the pheromone receptors, GprA/GprB, in turn influencing hyphal growth and promoting sexual development (Yu *et al*., 1996). GanB represents the putative carbon sensing system, upstream of cAMP-PKA pathway (Lafon *et al*., 2005). However, the GanB interacting GPCR(s) are unknown. Alternatively, the remaining characterised GPCR, GprD is proposed to interact with an uncharacterised Gα, upstream of the PKC pathway (Yu and d'Enfert, 2008). GprH influenced carbon and nitrogen sensing, in turn cAMP levels, PKA activity, glucose uptake and hyphal growth, reminiscent of an interaction with GanB. However, GprH also impacted on sexual development. Therefore, the interacting G-proteins for the two characterised putative nutrient sensors, GprD and GprH, remain to be determined.

The cAMP-PKA pathway performs a fundamental role in the regulation of fungal growth, metabolism and conidiation. Subsequently, the functions of components of the cAMP-PKA pathway are well characterised in multiple fungi (Shimizu and Keller, 2001; Banno *et al*., 2005; Freitas *et al*., 2010; Giacometti *et al*., 2011; Brown *et al*., 2014). In *A. nidulans*, the absence of the adenylate cyclase, CyaA, which generates the cAMP signal that activates the PKA complex, causes defects in conidial germination, hyphal growth and conidiation (Fillinger *et al*., 2002). In *A. nidulans*, the PKA complex consists of a regulatory subunit and two catalytic subunits. Similar to CyaA, the major catalytic subunit, PkaA, is required for proper conidial germination and hyphal growth while repressing conidiation (Shimizu

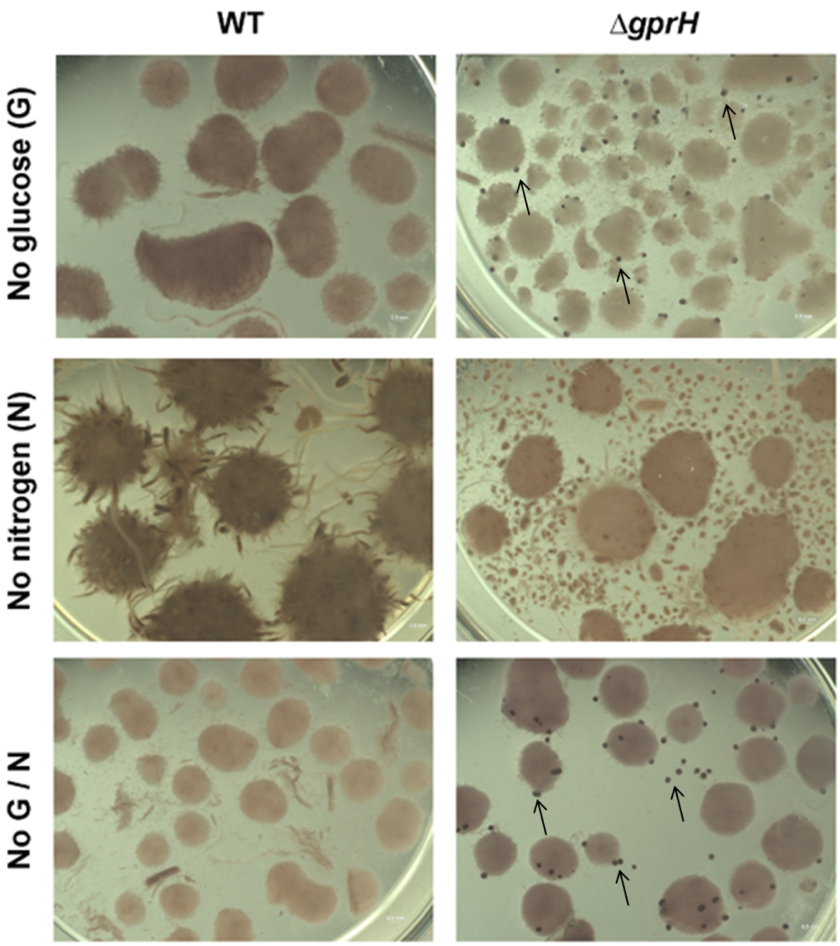

Fig. 9. GprH represses sexual development during carbon starvation. Post-8 days carbon starvation in submerged cultures (liquid media) which normally inhibit sexual development, the Δ*gprH* strain demonstrated a dramatically enhanced formation of cleistothecia. This response in the Δ*gprH* strain was specific to carbon starvation and did not occur in the absence of nitrogen. Arrows show cleistothecia.

and Keller, 2001; Lafon *et al*., 2005). In contrast, the effects of inactivating the regulatory subunit, PkaR, mirrored the effects of PkaA inactivation, with conidial germination in the absence of carbon and defects in conidiation (Yu and d'Enfert, 2008).

In *A. nidulans*, GprD is structurally related to the *S. cerevisiae* Gpr1 glucose receptor, which functions upstream of cAMP-PKA signalling (Han *et al*., 2004; de Souza *et al*., 2013). The absence of *gprD* in *A. nidulans* resulted in a delay in germination and growth defects (Han *et al*., 2004). However, evidence of a connection between GprD, a G-protein α subunit, and the cAMP-PKA pathway is lacking, while GprD has been implied to function upstream of PKC signalling (Han *et al*., 2004). In the present study, GprH also demonstrated homology to characterised fungal glucose and cAMP receptors. Both GprD and GprH are present in the conidia (Han *et al*., 2004; de Souza *et al*., 2013), possibly contributing to the absence of a delay in Δ*gprH* germination, comparable with Δ*gprD* strain in the presence of glucose. However, during prolonged carbon starvation, *gprH* and not *gprD* is significantly induced. Under these conditions, the absence of GprH resulted in a reduction in germling growth and an increasingly distinct metabolome. Additionally, the Δ*gprH* strain showed reduced glucose uptake, a process that is regulated by the cAMP-PKA pathway (Brown *et al*., 2014). Therefore, GprD and GprH appear to perform similar functions under distinct environmental conditions, with GprH regulating the cAMP-PKA pathway, metabolism and growth during carbon limitation.

Beyond primary metabolism and germination, environmental factors also influence sexual development. Sexual and asexual reproduction represents two competing developmental processes (Dyer and O'Gorman, 2012). In *A. nidulans*, sexual development normally requires well-nourished conditions, which is in contrast to nutrient limitation that triggers sex in other fungi (Zonneveld, 1977; Dyer *et al*., 1992). Sexual development in *A. nidulans* is favoured when the carbon : nitrogen ratio is balanced and a suitable amino acid source is present (Zonneveld, 1977; Han *et al*., 2004), while carbon and amino acid starvation inhibit sex (Hoffmann *et al*., 2000). The absence of *gprD* caused a dramatic increase in cleistothecia formation and inhibited conidial development (Han *et al*., 2004). Therefore, GprD plays a central role in hyphal growth and in the

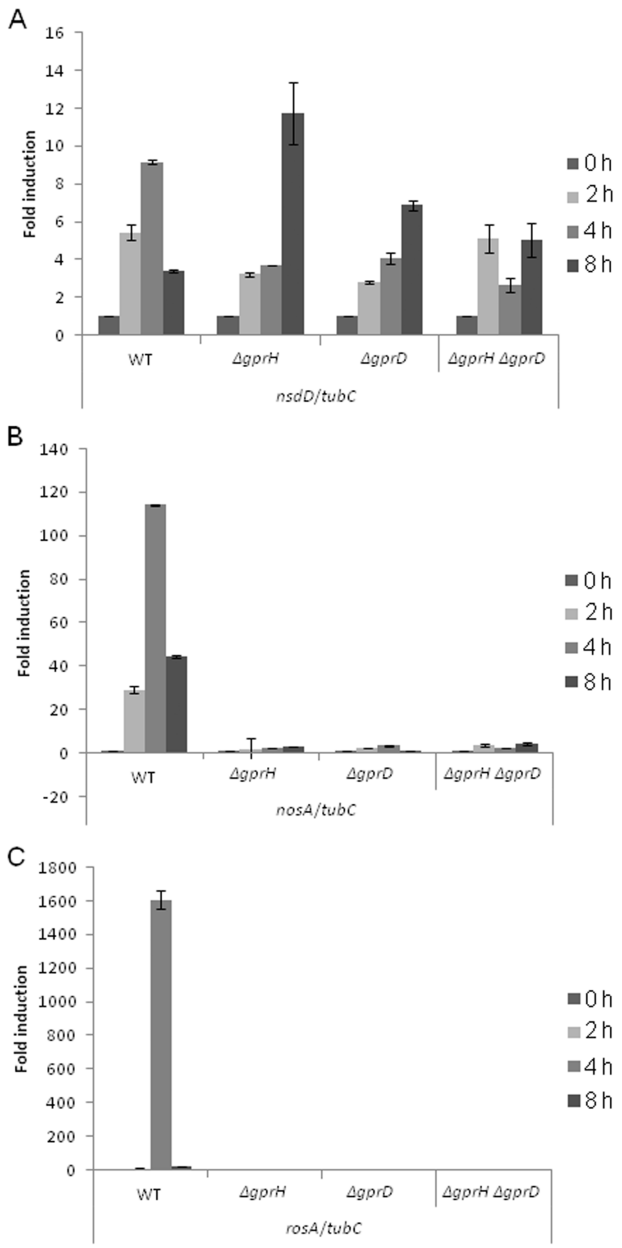

Fig. 10. GprH regulates the expression of transcription factors involved in sexual development post-carbon starvation. The wild-type, Δ*gprH*, Δ*gprD* and the Δ*gprH* Δ*gprD* strains were grown in minimal media containing 1% glucose (0 h) and then transferred to minimal media containing no carbon for 2, 4 and 8 h. RT-qPCRs show altered transcription of transcription factors in the absence of GprH and GprD in response to carbon starvation. The abundance of the respective mRNAs was normalised using *tubC*. Presented is the fold change in normalised mRNA abundance post-transfer from glucose to the respective carbon starvation condition.
A. Fold induction of *nsdD*.
B. Fold induction of *nosA*.
C. Fold induction of *rosA*.

inhibition of sexual development. Intriguingly, GprD-mediated nutrient sensing is postulated to function upstream of GprA/GprB pheromone signalling (Seo *et al*., 2004), suggesting that the requirement for suitable environmental conditions is paramount for sex to occur. Similar to GprD, the putative glucose and amino acid receptor, GprH, also repressed sexual development under conditions inductive of cleistothecia formation. However, distinct form GprD, the absence of GprH also resulted in dramatically enhanced sexual development during carbon starvation in submerged cultures. Intriguingly, a strain lacking both GprH and GprD did not produce cleistothecia in liquid medium during carbon starvation, implying that the two putative nutrient receptors, GprH and GprD, interact during carbon starvation to regulate sexual development.

The GATA-type transcription factor NsdD is crucial for cleistothecia formation under conditions inductive of sexual development, while *nsdD* over-expression resulted in the dramatic increase in cleistothecia on solid media and the formation of Hülle cells in submerged cultures that normally inhibit sexual development (Han *et al*., 2001). Two additional transcription factors, NosA and RosA, are also involved in the regulation of sexual development in *A. nidulans*. RosA is a negative regulator of sexual development, which is expressed at a very low level and is transiently upregulated during carbon starvation and post-12 h asexual development. NosA is constitutively expressed and is upregulated during late asexual development and upon carbon starvation (Vienken and Fischer, 2006). These two transcription factors are genetically dependent, with RosA repressing the expression of *nosA*. The Δ*rosA* strain demonstrated a slight increase in cleistothecia formation under standard conditions. However, Δ*rosA* promoted sexual development under low-glucose conditions and Hülle cells in submerged culture (Vienken and Fischer, 2006). Therefore, RosA represses sexual development post the integration of several environmental signals, while the interplay between RosA and NosA determines whether vegetative hyphae undergo sexual development (Vienken and Fischer, 2006).

The absence of GprH demonstrated a phenotype resembling the Δ*rosA* and *nsdD* overexpression strains, suggesting that GprH may also act as a regulator of NsdD, RosA, NosA function. The increased production of cleistothecia in the absence of GprD, under condition normally inductive of sexual development, was implicated to be the result of the derepression of *nsdD* expression (Han *et al*., 2004). In contrast, here, the absence of GprH and not GprD resulted in increased cleistothecia production in submerged cultures during carbon starvation, a condition normally inhibitive of sexual development. The Δ*gprH* strain did not show a dramatic increase in *nsdD* expression (except at 8 h), but did result in the loss of the

ability to induce *nosA* and *rosA* expression during starvation. The absence of the RosA repressor in the Δ*gprH* strain may therefore indicate an additional mechanism by which GprH regulates sexual development in response to carbon starvation.

In conclusion, GprH represents a putative glucose and tryptophan nutrient detection system that functions upstream of the cAMP-PKA pathway, influencing primary metabolism and hyphal growth while repressing sexual development via the RosA pathways in *A. nidulans*. GprH is pre-formed in conidia and is increasingly active during carbon starvation, where it performs a role in glucose uptake and the recovery of hyphal growth. Sexual reproduction generates genetic variation at a faster rate than asexual reproduction, quickening adaptation to the environment and improving survival. In *A. nidulans*, the increase in fitness associated with sex has been shown (Schoustra *et al.*, 2010). In addition, the thickened cell wall of sexual ascospores, compared with asexual conidia, enhances the survival of harsh environmental stresses (Dijksterhuis, 2007). However, sexual reproduction represents a dramatic energetic commitment. Therefore, GprH inhibits this unrequired developmental programme when conditions are not permissive.

Experimental procedures

Strains and culture conditions

The *A. nidulans* R21 wild-type strain (*pabaA4; yA1; veA1*) was used as reference in all experiments. The Δ*gprH* prototrophic mutant was generated by replacing the ORF region of *gprH* with *argB*$^+$ in PW1 (*biA1*; *argB2*; *methG1*; *veA1*; Fungal Genetics Stock Center). The 5′ and 3′ regions of the *gprH* gene were fused with *argB*$^+$ by Double-Joint PCR (Yu *et al.*, 2004), and the final Δ*gprH* amplicon was used for transformation. Transformants were selected for their growth without the arginine supplement, and the deletion of *gprH* was verified by genomic DNA base PCR followed by restriction enzyme digestion as described (Yu *et al.*, 2004) and Southern blot analysis (Supporting Information Fig. S5) according to Sambrook *et al.* (1989). A deletion strain (H4-12; *biA1*; *argB2*; *methG1*; Δ*gprH::argB+*; *veA1*) was then sexually crossed with *RRAW16 (pyrG89*, *yA2*; *veA*$^+$), and among the progeny, three *gprH* prototroph strains were isolated and confirmed.

The Δ*gprD* prototrophic strain (*pyrG89*; Δ*gprD*::*AnipyroA*$^+$*pyroA4*; *veA*$^+$ *Afu-pyrG*$^+$) was generated by replacing the coding region of *gprD* with the marker *pyroA*$^+$ in tNJ36.1 [*pyrG89*; *pyroA4*; *veA*$^+$*pyrG*$^+$ (*AfupyrG* PCR product)]. The 5′ and 3′ regions of the *gprD* gene were fused with *pyroA*$^+$ by Double-Joint PCR (Yu *et al.*, 2004), and the final Δ*gprD* amplicon was used for transformation. Transformants were selected for their growth without pyridoxine. The deletion of *gprD* was verified by genomic DNA base PCR followed by restriction enzyme digestion as described (Yu *et al.*, 2004) and Southern blot analysis according to Sambrook *et al.* (1989).

In order to get the double mutant (Δ*gprH* Δ*gprD*), a Δ*gprHpiro*$^+$ mutant strain was constructed using the AGB551 strain as a genetic background (*nkuA*Δ*::argB*;*pyrG89*; *pyroA4*; *veA*$^+$; Bayram *et al.*, 2012). Briefly, about 1.0-kb region of the 5′-UTR and 3′-UTRs for the *gprH* target gene (pRS426 *gprH* 5'UTR Fw + *gprH pyro*_5'UTR Rv and *gprH pyro* 3'UTR Fw + pRS426426 *gprH* 3'UTR Rv) was PCR amplified, purified and used for yeast transformation. As a selective marker, the *A. fumigatus pyroA* gene (*pyro* Fw and *pyro* Rv) was *amplified*. The Δ*gprH* cassette was constructed by *in vivo* recombination in *S. cerevisiae* (Colot *et al.*, 2006). The external 5′-UTR Forward and 3′-UTR Reverse primers possessed cohesive ends with the vector pRS426, and the internal primers 5′-UTR R and 3′-UTR F contained cohesive ends with 5′ and 3′ sequence of *pyroA* gene. The whole Δ*gprH* cassette was PCR-amplified from genomic DNA extracted from recombinant *S. cerevisiae*, purified and used to transform *A. nidulans* AGB551 strain according to Osmani *et al.* (1987). *A. nidulans* transformant strains were scored for their ability to grow on minimal medium without pyridoxine (Δ*gprH*) and homologous integration confirmed by PCR. After, the mutant Δ*gprHpiro*+ strain was used as a background for deletion of the g*prD* gene. The deletion cassette Δ*gprD* was constructed similarly to Δ*gprH* except for the prototrophy marker which was *pyrG*$^+$ instead of *pyroA*$^+$. Transformants were scored for their ability to grow on minimal medium lacking pyridoxine, uridine and uracil and homologous integration confirmed by PCR.

All PCR reactions were performed using Phusion High-Fidelity DNA polymerase (New England Biolabs), except for the amplification of whole cassettes where *TaKaRa Ex Taq DNA Polymerase* (Clontech USA) was used. The strains used in this study are listed in Supporting Information Table S4, and all of them were propagated at 37°C in minimal media (1% w/v glucose, nitrate salts, trace elements, pH 6.5) or complete media (YG: 2% w/v glucose, 0.5% yeast extracts, trace elements) plus or minus agar (2% w/v), in addition to the respective supplements depending on the strains auxotrophy. All the primers used in this work are listed in Supporting Information Table S5.

Protein domain and phylogenetic analyses

Filamentous fungal homologues of GprH were identified via delta BlastP search of GprH (AN8262) against the NCBI database. The organisation of the GprH protein was assessed using the SMART interface (http://smart.embl-heidelberg.de/) and the Phyre2 protein structure prediction tool (Kelley and Sternberg, 2009). Homologues within *Aspergilli* and other prominent filamentous fungal systems, with protein identity greater than 1e-45, were selected (Supporting Information Table S1; GEO Number GSE42732). Protein alignment was performed in Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/), and the phylogenetic tree was built in MEGA6 (Tamura *et al.*, 2013) using the neighbour-joining method, and the bootstrap values were calculated from 500 replicates. Evolutionary distance was calculated using the Poisson correction method and represents the number of amino acid substitutions per site. The outgroup used consists of characterised filamentous fungal GPCRs, including the putative pheromone and sugar receptors in *A. nidulans* and *N. crassa*.

Glucose uptake assay

Glucose uptake rates were measured by assaying the incorporation of D-[U-^{14}C] glucose [289.0 mCi mmol^{-1} (10.693 GBq) mmol^{-1}] (Perkin Elmer Life Sciences) in germinating conidia at various D-glucose concentration according to dos Reis *et al*. (2013).

RNA extractions, Northern blot and RT-qPCR analyses

Two approaches were used to evaluate the transcriptional differences in the expression of genes involved in sexual development. Firstly, gene expression was monitored under conditions inductive of cleistothecia development (air-exposed + sealed plates) and secondly during carbon and nitrogen starvation in liquid media. Asexual spore development was synchronised by transferring a thin mycelial mat filtered from liquid YG culture grown for 24 h to an agar plate. To induce sexual development, air-exposed mycelia were incubated in sealed plates for 11 days (0–2 days: conidiophore development, asexual development; 2–11 days: cleistothecia development, sexual development; and 6–11 days: presence of ascospores). For the starvation experiment in liquid media, 1×10^7 conidia were incubated in minimal media containing 1% glucose as a sole carbon source at 37°C in a rotatory shaker (180 r.p.m.) for 24 h. Subsequently, mycelia were washed with sterile water and then incubated in a modified minimal media (AMM) without any carbon or nitrogen source at 37°C for an additional 12 or 24 h. For both experimental approaches, fungal biomass was harvested at the represented intervals and immediately frozen in liquid nitrogen. Total RNA was extracted using TRIAZOL (Invitrogen), treated with DNAse (Promega) and purified using RNeasy Plant Mini Kit (Qiagen) according to manufacturers' instructions. RNA integrity was confirmed using the Bioanalyser Nano kit (Agilent technologies) and the Agilent Bioanalyser 2100. Northern blot analysis was performed according to Sambrook *et al*. (1989). cDNA was synthesised from 5 μg of RNA using SuperScript III (Invitrogen). Quantitative PCR (qPCR) analyses were performed according to Semighini *et al*. (2002). The abundance of the respective mRNAs was normalised for fungal biomass using *tubC*. The primers for *gprB*, *gprD*, *gprH*, *nsdD*, *rosA* and *nosA* are listed in Supporting Information Table S5.

Metabolite extraction

The metabolite extraction protocol used was based on the procedure performed byde de Souza *et al*. (2013). After growth of *A. nidulans* wild-type and Δ*gprH* on minimal media containing 2% glucose (w/v) for 24 h at 37°C with shaking (180 r.p.m.), the mycelia was washed with sterile distilled water and transferred to minimal media without any carbon source for 0, 6 and 12 h at 37°C with shaking (180 r.p.m.). The mycelia was collected by vacuum filtration and immediately frozen. The metabolites were extracted from frozen tissues (0.1 g) that was ground into a fine powder in liquid nitrogen. To each powdered sample, 800 μl of extraction buffer containing 1:1 (v/v) acetonitrile-*d3* (CD_3CN) : deuterium oxide (D_2O) was added. Extracts were clarified by centrifugation at full speed for 2 min and the supernatants transferred to a new tube and immediately frozen for further processing to ^{1}H NMR analysis.

^{1}H NMR analysis

For NMR-based metabolomic analysis, dried media samples were resuspended in 70 μl of deuterium oxide (D_2O) with 20 mM phosphate buffer, 0.1 mM 2,2′,3,3′ deutero-trimethylsilylpropionate (TSP) and 1 mM formate. Proton (^{1}H) spectra were acquired at 25°C on a 14.1 T Varian INOVA spectrometer (600 MHz ^{1}H frequency) equipped with a CapNMR™ microcoil (Magnetic Resonance Microsensors Corp, Savoy, MN, USA). The ^{1}H NMR spectra were acquired using a one-pulse sequence with pre-saturation of the water resonance using a 90° flip angle and a total repetition time (TR) of 5.65 s. The time to acquire a spectrum from each sample was approximately 40 min with 512 transients.

Molar ratios were calculated from the ^{1}H NMR spectra by comparing peak areas to the total integration of each spectrum after removing the water, formate and TSP peaks. The spectral data of the culture media was processed using ACDLabs 12.0 1D NMR Processor (ACD Labs). Spectral processing followed a standard routine as previously described (de Souza *et al*., 2013). Briefly, prior to Fourier transformation, the spectra were zero-filled to 32,000 points, and an apodisation Gaussian function of 0.5 Hz was applied. Spectra were phased, baseline corrected, and reference to TSP set to 0 ppm and spectra was binned into 0.04 ppm segments using ACDLabs. Metabolites were identified by comparing NMR spectral identifiers (chemical shifts, peak area ratios, peak multiplicity, coupling constants) to metabolomic NMR databases [Human Metabolome Database (http://www.hmdb.ca); University of Wisconsin (http://www.bmrb.wisc.edu/metabolomics/)] and to known aspergillus metabolites (Ruijter *et al*., 2003; Diano *et al*., 2009; de Souza *et al*., 2013; Orosz *et al*., 2014). In order to determine the main contributions to variation in the data, PCA was conducted using pcaMethods in the Bioconductor package (Stacklies *et al*., 2007). The effects of the genotypes and treatment were assessed by ANOVA followed by Tukey's tests (*P* value) using the statistical software R (2015).

Microarray analyses

Initially, 1×10^7 conidia were incubated in liquid minimal media containing 1% glucose as a sole carbon source at 37°C in a rotatory shaker (180 r.p.m.) for 24 h. Subsequently, mycelia were washed with sterile water and then incubated in minimal media without any carbon source, at 37°C, for an additional 4 and 8 h. At each step, the mycelia were collected, in triplicate, by vacuum filtration and immediately frozen in liquid nitrogen. Agilent custom-designed oligonucleotides arrays (Krohn *et al*., 2014) were used to identify the transcriptional differences between growth on glucose (Cy3 reference) and carbon starvation (Cy5) for both the wild-type and Δ*gprH* strains. Total RNA was extracted using TRIAZOL (Invitrogen) and purified using RNeasy Plant Mini Kit (Qiagen) according to manufacturers' instructions. RNA integrity was confirmed using the Bioanalyser Nano kit (Agilent technolo-

gies) and the Agilent Bioanalyser 2100. Array hybridisation and data analysis were performed according to Krohn *et al.* (2014). The dataset was deposited in the Gene Expression Omnibus http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc (GSE65282).

Genes were determined as differentially expressed between the different nutritional conditions using a *t*-test ($p < 0.01$) performed within the Mev software (Saeed *et al.*, 2003). The differentially expressed genes were divided into those which were up or downregulated. The overrepresented GO terms within the differentially expressed gene sets from each strain, under the two nutritional conditions, were identified using the FetGOat (http://www.broadinstitute.org/fetgoat/index.html) software. FetGOat-assigned GO terms, which are supported by bioinformatic and in some cases experimental data, were used to describe gene functions.

cAMP and PKA activity assays

Initially, 1×10^7 conidia were incubated in liquid YG media at 37°C in a rotatory shaker (180 r.p.m.) for 16 h. Subsequently, mycelia were washed with sterile water and then incubated in a modified minimal media (AMM) lacking glucose, hence containing no carbon source, at 37°C for 4 h. Finally, the media was replaced with AMM plus glucose (2% w/v) and incubated for the represented intervals at 37°C. Extraction of cAMP and PKA was performed according to Fillinger *et al.* (2002) and Brown *et al.* (2013) respectively. The Amershan cAMP Biotrak EIA system assay (GE Healthcare) and the Peptag cAMP-dependent PKA activity assay (Promega) were performed according to manufacturer's instructions. Total protein content in the respective extracts was measured using the Bio-Rad protein assay according to manufacturer's instructions. PKA activity was quantified via measuring the intensity of the phosphorylated substrate using the ImageJ software (Schneider *et al.*, 2012). The cAMP and PKA results are presented as the cAMP concentration or PKA activity per μg total protein.

Microscopy and germling growth post-carbon starvation

Conidia were incubated in minimal media for 4 h at 30°C and then transferred to the modified minimal media without any carbon or nitrogen source for 4 h. Finally, the starved germlings were returned to minimal media for an additional 1 h. Prior to examination, coverslips were washed once in phosphate buffered saline (140 mM NaCl, 2 mM KCl, 10 mM $NaHPO_4$, 1.8 mM KH_2PO_4, pH 7.4). Finally, germlings were examined using a Zeiss epifluorescence microscope, and the phase contrast bright-field images were captured with AxioCam camera and processed using the AxioVision software version 3.1 (Carl Zeiss).

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