RESEARCH ARTICLE

Characterization of gprK Encoding a Putative Hybrid G-Protein-Coupled Receptor in Aspergillus fumigatus

Mun-Gu Jung¹, Sung Su Kim², Jae-Hyuk Yu³*, Kwang-Soo Shin¹*

¹ Department of Life Science, Daejeon University, Daejeon, Republic of Korea, ² Department of Biomedical Laboratory Science, Daejeon University, Daejeon, Republic of Korea, ³ Departments of Bacteriology and Genetics, University of Wisconsin-Madison, Madison, Wisconsin, 53706, United States of America

* shinks@dju.kr (KSS); jyu1@wisc.edu (JHY)

Abstract

The G-protein-coupled receptor (GPCR) family represents the largest and most varied collection of membrane embedded proteins that are sensitized by ligand binding and interact with heterotrimeric G proteins. Despite their presumed critical roles in fungal biology, the functions of the GPCR family members in the opportunistic human pathogen Aspergillus fumigatus are largely unknown, as only two (GprC and GprD) of the 15 predicted GPCRs have been studied. Here, we characterize the gprK gene, which is predicted to encode a hybrid GPCR with both 7-transmembrane and regulator of G-protein signaling (RGS) domains. The deletion of gprK causes severely impaired asexual development coupled with reduced expression of key developmental activators. Moreover, ΔgprK results in hyper-activation of germination even in the absence of carbon source, and elevated expression and activity of the protein kinase A PkaC1. Furthermore, proliferation of the ΔgprK mutant is restricted on the medium when pentose is the sole carbon source, suggesting that GprK may function in external carbon source sensing. Notably, the absence of gprK results in reduced tolerance to oxidative stress and significantly lowered mRNA levels of the stress-response associated genes sakA and atfA. Activities of catalases and SODs are severely decreased in the ΔgprK mutant, indicating that GprK may function in proper activation of general stress response. The ΔgprK mutant is also defective in gliotoxin (GT) production and slightly less virulent toward the greater wax moth, Galleria mellonella. Transcriptomic studies reveal that a majority of transporters are down-regulated by ΔgprK. In summary, GprK is necessary for proper development, GT production, and oxidative stress response, and functions in down-regulating the PKA-germination pathway.

Introduction

Heterotrimeric G protein signal transduction is conserved in all eukaryotes and is crucial for sensing and responding to external signals including nutrients, physicochemical stimuli, and environmental stress. A canonical heterotrimeric G-protein system is composed of a
7-transmembrane (TM) domain G-protein-coupled receptor (GPCR), a heterotrimeric G protein consisting of α, β, and γ subunits, and a down-stream effector [1–4]. GPCRs are sensitized by binding of ligand(s), which changes the GPCR’s interaction with heterotrimeric G proteins. Inactive G proteins are activated via GDP-GTP exchange at the Gα subunit, which results in the dissociation of GTP-Gα from the Gβγ heterodimer. Once dissociated, GTP-Gα, Gβγ, or both can elicit and propagate signals by modulating activities of a number of downstream effectors. Signaling is turned off when GTP is hydrolyzed to GDP by the intrinsic GTPase activity of a Gα subunit, resulting in the reformation of an inactive GDP-Gαβγ heterotrimer [2, 4]. Key components in modulating G-protein signal transduction are the regulators of G protein signaling (RGS), which assist in quenching the signal by accelerating the intrinsic GTPase activity of the Gα subunit. Signaling can also be turned off by internalization of the GPCR, triggering endocytosis and degradation of the receptor [5].

Based on their potential importance to fungal growth and survival and as potential drug targets, GPCRs have been the subject of numerous bioinformatics studies. As a result, 15 putative GPCRs were identified in *Aspergillus fumigatus*, an opportunistic human pathogenic fungus, which causes allergy and invasive pulmonary aspergillosis (IPA) in immune-compromised patients [6–8]. These GPCRs have been assigned to nine groups based on their phylogenetic relationship with those found in *A. nidulans* [6, 9–11]. The two putative pheromone receptors GprA (AFUA3G14330) and GprB (AFUA5G07880) are grouped to Classes I and II, respectively. The Class III GPCR GprC (AFUA7G04800) and GprD (AFUA2G12640), which have high similarity to the *Saccharomyces cerevisiae* Gpr1 [12, 13], might be involved in carbon-sensing. GprF (AFUA5G04100), GprG (AFUA1G19000), and GprJ (AFUA1G06840) belong to Class IV and might function in nitrogen-sensing, and they are similar to the *Schizosaccharomyces pombe* Stm1 [14]. Class V includes the three putative cAMP receptors GprH (AFUA5G04140), GprI (AFUA3G00780), and GprL (AFUA3G01750), which are similar to the *Dictyostelium discoideum* cAMP receptor cAR1 [6, 10]. GprK (AFUA4G01350) has both a 7-TM and an RGS domain and belongs to Class VI and might function in nitrogen-sensing, and they are similar to the *Schizosaccharomyces pombe* Gpr1 [12, 13]. Class VI includes GprH (AFUA1G06840) and GprL (AFUA3G01750), which are similar to the *Saccharomyces cerevisiae* Gpr1 [12, 13]. Class VII includes GprM (AFUA7G05300), similar to rat growth hormone-releasing factor receptors [15]. Class VIII includes GprO (AFUA3G10570) and GprP (AFUA6G07160), similar to yeast Izh zinc regulators [16, 17]. Finally, Class IX is comprised of a single GPCR, NopA (AFUA7g01430), which is similar to bacterial opsins [6].

Previously we characterized functions of GprA, GprB, and GprD in *A. nidulans* [9, 18]. The gprA and gprB genes encode putative GPCRs similar to the yeast pheromone receptors Ste2p and Ste3p, respectively [18]. GprA and GprB are specifically required for self-fertilization. Deletion of gprA and/or gprB results in formation of reduced numbers of cleistothecia that are smaller than those of wild type and carry few viable ascospores. GprD is involved in the positive regulation of germination and negative control of sexual development in *A. nidulans* [9]. Deletion of gprD results in delayed conidial germination and enhanced sexual development [9]. In addition, the GprD homologue mediates the increase of intracellular cAMP in response to oxygenated polyunsaturated fatty acids (oxylipins), which act as autocrine and paracrine mediators in human [19].

Only two GPCR-like proteins of *A. fumigatus* have been characterized [20]. GprC and GprD that are homologs of Gpr1p in *S. cerevisiae* activate the cAMP pathway in response to glucose [13, 21]. Deletion of *A. fumigatus* gprC or gprD resulted in impaired growth and severely attenuated virulence [20]. In this study, we have investigated the roles of the gprK gene in growth, differentiation, nutrient sensing, stress response, and virulence in *A. fumigatus*. Results indicate that the putative hybrid GPCR-RGS protein GprK might function as a key upstream controller, governing multiple biological processes in this important human pathogenic fungus.
Materials and Methods
Strains, media, and culture conditions
Aspergillus fumigatus strains were grown on YPD or MMG and 0.1% yeast extract (YE) at 37°C as previously described [22]. For auxotrophic mutants, the medium was supplemented with 5 mM uridine and 10 mM uracil [23]. For liquid submerged culture, about 5 × 10⁵ conidia/ml were inoculated into liquid MMG with 0.1% YE and incubated at 37°C. For phenotypic analyses of A. fumigatus strains on air-exposed culture, conidia (1 × 10⁶) of relevant strains were spotted on solid medium and incubated at 37°C for 3 days. To examine development and secondary metabolite production in liquid submerged culture, spores of relevant strains were inoculated to a final concentration of 5 × 10⁵ conidia/ml in 50 ml of liquid MMG with 0.1% YE and incubated at 250 rpm at 37°C for 4 days.

Generation of the gprK null mutant
The oligonucleotides used in the present study are listed in S1 Table. The gprK gene was deleted in A. fumigatus AF293.1 (pyrG1) strain [23] by employing double-joint PCR (DJ-PCR) [24]. The deletion construct containing the A. nidulans selective marker (AnipyrG⁺) with the 5' and 3' flanking regions of the gprK gene was introduced into the recipient strain AF293.1 [25]. The selective marker was amplified from FGSCA genomic DNA with the primer pair oligo 109/oligo 110. The gprK mutant was isolated and confirmed by PCR, followed by restriction enzyme digestion [24]. To complement ΔgprK, a single joint PCR (SJ-PCR) method was used [24]. The gprK gene's ORF with presumed promoter and terminator was amplified with specific primer pairs where the 3' reverse primer carries overlapping sequences with the ptrA gene's 5' end. Amplification of the ptrA gene was carried out with primer pairs where the 5' forward primer carries overlapping sequences with gprK gene's 3' end. The final amplicon was amplified with the nested primer pair oligo 786/oligo 731 and introduced into a ΔgprK strain by transformation. To complement ΔgprK with the GPCR domain alone without RGS, the genomic region of gprK covering the predicted GPCR region along with the presumed promoter and terminator was amplified with the primer pair (oligo 295/oligo 886). Amplification of the ptrA gene was carried out with the primer pairs where the 5' forward primer carries overlapping sequences with the gprK gene's 3' end. The final fusion construct was amplified with the nested primer pair oligo 889/oligo 890 and introduced into a ΔgprK strain.

Nucleic acid isolation and manipulation
To isolate genomic DNA from A. fumigatus, about 10⁶ conidia were inoculated in 2 ml of liquid MMG + 0.5% YE, and stationary cultured at 37°C for 24 h. The mycelial mat was collected and squeeze-dried, and genomic DNA was isolated as described [24]. The deletion mutant was confirmed by PCR amplification of the coding region of the gene followed by restriction enzyme digestion of the PCR amplicon.

Total RNA isolation was carried out as previously described [9, 26]. Briefly, conidia (5 × 10⁵ conidia/ml) of WT, ΔgprK and complement strains were inoculated into liquid MMG with 0.1% YE and incubated at 37°C, 250 rpm. Individual mycelial samples were collected at designated time points from liquid submerged cultures and squeeze-dried. The sample was homogenized using a Mini Bead beater in the presence of 1 ml of TRizol® reagent (Invitrogen) and 0.3 ml of silica/zirconium beads (Biospec). RNA extraction was performed according to the manufacturer’s instruction (Invitrogen). Quantitative RT-PCR (qRT-PCR) assays were performed according to the manufacturer's instruction (Qiagen, USA) using 96-well optical plates and a Rotor-Gene Q (Qiagen, USA). Each run was assayed in triplicate in a total volume of
20 μl containing the RNA template, One Step RT-PCR SYBR Mix (Doctor Protein, Korea), reverse transcriptase, and 10 pmole of each primer (S1 Table). Reverse transcription was performed at 42°C for 30 min. PCR conditions were 95°C/5 min for one cycle, followed by 95°C/30 s and 55°C/30 s for 40 cycles. Amplification of one single specific target DNA was checked by melting curve analysis (+0.5°C ramping for 10 s, from 55°C to 95°C). The expression ratios were normalized to EF1α expression and calculated according to the ΔΔCt method [27].

Phenotypic analyses

Germination rates were measured as previously described with a slight modification [28]. To examine germination levels, conidia of WT and mutant were inoculated in 5 ml of liquid MMG with 0.1% YE, or liquid medium lacking a carbon source, and incubated at 37°C. Levels of germination were examined every 2 h after inoculation under a microscope.

To assess the effects of a variety of carbon sources (1%), strains were grown on a variety of media, the base being MMG as described above. About 1×10⁶ spores of each strain in 0.1% Tween 20 were inoculated and incubated at 37°C for 3 days with three replicate plates per condition.

Various media were used to assess the roles of GprK in stress responses. For oxidative stress test, hydrogen peroxide (10 mM), menadione (100 μM), and paraquat (100 μM) were added to the YG media after autoclaving and cool down. The following stressors were added to the YG media after autoclaving to assess cell wall stress and osmotic stress: Congo red (100 μg/ml), calcofluor white (100 μg/ml), caspofungin (0.2 μg/ml), sodium chloride (1 M), and sorbitol (1 M). To assess pH stress, media were buffered to three different pH levels (4.0, 6.0, and 8.0) before autoclaving.

To assess the production of gliotoxin (GT), conidia of each strain were inoculated into 50 ml liquid MMY and incubated for 7 days at 37°C and 280 rpm. GT was extracted with chloroform as described previously [29]. The chloroform extracts were air-dried and resuspended in 100 ml of methanol. Ten μl aliquots of each sample were applied to a thin-layer chromatography (TLC) silica plate containing a fluorescence indicator (Kiesel gel 60, E. Merck). The TLC plate was developed with toluene:ethyl acetate:formic acid (5:4:1, v/v) until the solvent front reached about 15 cm. GT standard was purchased from Sigma (USA).

PKA, SOD, and catalase assay

A. fumigatus strains were grown in MMG with 0.1% YE for 24 h at 37°C. Mycelia were suspended in the lysis buffer (50 mM Tris-HCl pH 7.2, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA) and homogenized using a Mini Bead-Beater (BioSpec Products). The homogenate was centrifuged in a microcentrifuge for 5 min at 15,000 rpm at 4°C, and the supernatant (10 μl, 3 mg/ml) was used in assay for PKA activity using fluorescent dye-coupled kemptide peptide (Promega, USA) as the photoacceptor according to the manufacturer’s instructions. For catalase and SOD activity assays, protein was extracted from the conidia of WT, mutant, and complement strains. Conidia of each strain were suspended in the lysis buffer and homogenized using a Mini Bead-Beater (BioSpec Products). The homogenate was centrifuged in a microcentrifuge for 5 min at 15,000 rpm at 4°C, and the supernatant was used for further analyses. For the detection of catalase activity on gels, the mycelial extracts were subjected to non-denaturing PAGE, and the ferricyanide-negative stain was used to locate bands containing catalase activity [30]. SOD activity on a gel was visualized by inhibition of the reduction of NBT (Sigma) according to the method of Beauchamp and Fridovich [31].

Virulence assay

Conidia invasion assay was performed with the type II human alveolar cell line A549. A549 cells were seeded in 24-well plates at a concentration of 1 × 10⁵/well and incubated at 37°C for 1 h. Conidia suspension (100 μl) was added to the cells at a multiplicity of infection (MOI) of 10.
and incubated for 4 h at 37°C. The cells were then washed five times with PBS, and Triton X-100 (200 μl/well) was added to the well in order to disrupt the cells and release the intracellular conidia. After harvesting the conidia by centrifugation, the conidia were resuspended in 600 μl PBS and 100 μl aliquots of the resuspended conidia were incubated on MMG at 37°C for 36 h.

The insect survival assay was performed as previously described with some modifications [32]. Briefly, sixth instar G. mellonella used for experiments were selected to be similar in size (approximately 0.3 g). Larvae were infected by injecting the fresh conidia (1 × 10^5, 5 μl) into the last left pro-leg. The petri dishes were stored in a container loosely covered with aluminum foil and incubated at 37°C in the dark for the duration of the experiment. Larvae were checked daily for survival and larvae that did not respond to stimulation were recorded as dead. The larvae were fixed by immersion in 10% (v/v) neutral buffered formalin for 3–7 days. The larvae were blocked by a longitudinal section and sections were embedded in paraffin wax. Thin sections of the paraffin-embedded tissue were then stained with hematoxylin and eosin (H&E) and Periodic acid-Schiff (PAS) for microscopic examination.

Microarray analysis
For control and test RNAs, the synthesis of target cDNA probes and hybridization were performed using Agilent’s Low Input Quick Amp WT Labeling Kit (Agilent Technology, USA) according to the manufacturer’s instructions. Briefly, 100 ng total RNA was mixed with WT primer mix and incubated at 65°C for 10 min. cDNA master mix (5× First strand buffer, 0.1 M DTT, 10 mM dNTP mix, RNase-Out, and MMLV-RT) was prepared and added to the reaction mixture. The samples were incubated at 40°C for 2 h, and then the RT and dsDNA synthesis reactions were terminated by incubating at 70°C for 10 min. The transcription master mix was prepared as directed by the manufacturer’s protocol (4× Transcription buffer, 0.1 M DTT, NTP mix, 50% PEG, RNase-Out, inorganic pyrophosphatase, T7-RNA polymerase, and Cyanine 3/5-CTP). Transcription of dsDNA was performed by adding the transcription master mix to the dsDNA reaction samples and incubating at 40°C for 2 h.

Amplified and labeled cRNA was purified on an RNase mini column (Qiagen) according to the manufacturer’s protocol. Labeled cRNA target was quantified using a ND-1000 spectrophotometer (NanoDrop Technologies, USA). After checking labeling efficiency, each of cyanine 3-labeled and cyanine 5-labeled cRNA target were mixed, and fragmentation of cRNA was performed by adding 10× blocking agent and 25× fragmentation buffer and incubating at 60°C for 30 min. The fragmented cRNA was resuspended with 2× hybridization buffer and directly pipetted onto assembled MYcroarray.com (A. fumigatus AF293) 30K Microarray. The arrays hybridized at 57°C for 17 h using an Agilent Hybridization oven (Agilent Technology, USA). The hybridized microarrays were washed as per the manufacturer’s washing protocol (Agilent Technology, USA). Hybridization images were analyzed by an Agilent DNA microarray Scanner (Agilent Technology, USA), and the data quantification was performed using Agilent Feature Extraction software 10.7 (Agilent Technology, USA). The average fluorescence intensity for each spot was calculated and local background was subtracted using Gene Pix Pro 6.0 (Axon Instruments, USA). Loess normalization and selection of fold-changed genes were performed using GenoWiz 4.0 (Ocimum biosolutions, India). The data is available in the Gene Expression Omnibus (GEO) at NCBI (the accession number = GSE83200).

Results
Summary of A. fumigatus GprK
Based on their domain structures, GprK was aligned and compared with other putative GPCRs in A. fumigatus (S1A Fig). GprK consists of 559 amino acids and contains a 7-TM domain, 2
low compositional complexity regions, and an RGS domain at the C-terminal end. This RGS domain consists of 174 amino acids and starts at position 372 and ends at position 545 (E-value; 1.9e-8). This GprK and its potential homologs in Neosartorya fischeri (A. fischerianus), A. clavatus, A. niger, A. oryzae, A. flavus, A. terreus, and A. nidulans were aligned and a phylogenetic tree was generated (S1B Fig). GprK of A. fumigatus shares 47 and 62.5% identity with the hypothetical protein of A. niger CBS 513.88 (An04g07760) and GprK of A. nidulans FGSC A4 (AN7795), respectively (S1C Fig).

GprK is required for proper asexual development

To characterize functions of GprK in asexual development, we generated the ΔgprK mutant by replacing the ORF with the A. nidulans pyrG marker. We also generated complemented strains (C') via re-introducing the wild type (WT) allele of gprK to a deletion strain. As shown in Fig 1A, the radial growth rate of the ΔgprK mutant colony on solid medium was similar to those of WT and C' strains. Although the gprK deletion strain demonstrated no change in radial growth, it formed very faint colonies with highly reduced thallic density and significantly lowered formation of conidiophores compared to WT and C' strains (Fig 1A). Moreover, whereas the colony edge of WT and C' strains contained an abundance of conidiophores, the ΔgprK mutant exhibited a relatively small number of conidiophores (Fig 1A, right panels). Quantitative analyses of conidia per plate grown on solid medium further demonstrated that asexual spore production in the ΔgprK mutant (0.50 × 10^{10} conidia/plate) was dramatically decreased to a level that was only about 25% of WT and C' strains (Fig 1B). We then analyzed mRNA levels of key asexual developmental regulators, abaA, brlA, vosA, and wetA, and found that the deletion of gprK resulted in significantly reduced levels of their mRNA at all times tested (Fig 1C). These results suggest that GprK is necessary for both proper control of conidiation on air-exposed solid culture and for developmental regulator expression. To check whether the GPCR domain alone in GprK is sufficient to restore the growth and developmental phenotypes, we generated five individual strains with the GPCR domain alone in the gprK null background (ΔgprK::gpcr+) and analyzed their phenotypes. As shown in Fig 1D, while ΔgprK::gpcr+ strains exhibited the faint and low-thallic density phenotype like the ΔgprK mutant, they all showed enhanced radial growth compared to WT, C', and mutant strains. These results indicate that both GPCR and RGS domains are necessary for the full functionality of GprK, and its GPCR domain without the RGS domain may alter the control of vegetative growth.

GprK downregulates spore germination and cAMP signaling pathway

To investigate a potential role of GprK in controlling spore germination, we analyzed the kinetics of germ tube emergence in the ΔgprK mutant in comparison to that of WT and C' strains. In the presence of glucose, conidia of all strains began to germinate at 6 h of incubation. However, at 8 h, while ~35% of WT and C' strains conidia germinated, 80% of the ΔgprK mutant conidia germinated, suggesting that GprK may negatively regulate conidial germination. This was more evident in the absence of carbon source, where the germination time and rate of ΔgprK strain conidia germination were more rapid and higher than those of WT and C' strains (Fig 2A).

Previous studies have demonstrated that the heterotrimeric G-protein composed of GanB, SfaD, and GpgA activates a cAMP-dependent protein kinase (PKA) pathway in response to glucose, whose signaling is attenuated by RgsA in A. nidulans [33–35]. In A. nidulans, PkaA is the primary PKA that positively functions in vegetative growth and spore germination but negatively controls asexual sporulation [28]. As the deletion of gprK resulted in hyper-active germination and reduced conidiation in A. fumigatus, we tested whether this deletion altered
Multiple Roles of Aspergillus GprK
Fig 1. The role of GprK in asexual development. (A) Colony photographs of WT (AF293), ΔgprK, and complemented (C) strain point-inoculated on solid MMG with 0.1% YE and grown for 3 days (Top: left; Bottom: middle panels). The enlarged photographs from the plate (indicated by the white box) are shown in the right panels with the bar indicating 1 mm. (B) Conidia numbers produced by each strain per plate. Student’s t-test: *p < 0.05, **p < 0.01. (C) mRNA levels of the asexual developmental regulator genes in WT, ΔgprK, and C’ strains determined by quantitative PCR (qRT-PCR). Cultures were incubated in liquid MMY and mRNA levels were normalized using the ef1α gene, according to the ΔΔCt method. Data are expressed as the mean ± standard deviation from three independent experiments. Student’s t-test: *p < 0.05, **p < 0.01. (D) Photographs of colonies of WT, ΔgprK, C’, and ΔgprK: gpcr+ strains point-inoculated on solid MMG and grown for 3 days (Top: left; Bottom: right panels).

doi:10.1371/journal.pone.0161312.g001

Fig 2. The role of GprK in spore germination and PKA activity. (A) Kinetics of germ outgrowth in A. fumigatus strains when inoculated in liquid MMG at 37°C in the presence or absence (“no C”) of glucose. (B) Accumulation of pkaC1 mRNA in WT, ΔgprK, and C’ strains analyzed by qRT-PCR. Student’s t-test: **p < 0.01. (C) PKA activity of A. fumigatus strains as monitored by gel electrophoresis. A phosphorylated substrate migrates toward the cathode (+). Each strain was grown in MMG for 24 h at 37°C, at which time a mycelial extract was analyzed. Note that the expression of pkaC1 mRNA and PKA activity were significantly increased in the ΔgprK mutant strain compared to WT and C’ strains.

doi:10.1371/journal.pone.0161312.g002
levels of PkaC1, a major catalytic subunit of PKA in *A. fumigatus*. As shown in Fig 2B, pkaC1 mRNA levels were about two-fold higher in the ΔgprK mutant than in WT and C' strains. PKA activity was assessed using colored kemptide, a peptide substrate specifically recognized and phosphorylated by PKA. As shown in Fig 2C, WT and C' strains exhibited very little PKA activity until cAMP was added. In contrast, PKA activity in the ΔgprK strain was clearly detectable even in the absence of cAMP, and the activity increased about 10-fold in the presence of cAMP. These results indicate that GprK may negatively control cAMP-dependent signaling pathway, which could explain the apparent developmental and germination phenotypes of the ΔgprK mutant.

**GprK functions in carbon source sensing and oxidative stress response**

The presence or absence of carbon and nitrogen sources can be detected by various fungal GPCRs, including Gpr1 of *S. cerevisiae* [12, 13, 36] and GPR-4 of *Neurospora crassa* [37]. However, a nutrient-sensing GPCR has not been identified in the aspergilli except in *A. flavus* [38]. The ΔgprA, ΔgprC, ΔgprL, ΔgprK, and ΔgprR mutants of *A. flavus* were growth impaired on several carbon sources [38]. To investigate a potential role for the *A. fumigatus* GprK in carbon source sensing, the ΔgprK strain was grown on a variety of carbon sources such as arabinose, galactose, glucose, ribose, sucrose, and xylose. As shown in Fig 3A, while there were no differences in growth on hexoses, the ΔgprK mutant was severely growth restricted with arabinose or ribose as the carbon source, suggesting that GprK may play a role in pentose sensing.

We then asked whether GprK is associated with stress responses by exposing the ΔgprK mutant to a variety of stressors and measuring the radial growth. Distinct from the ΔgprK mutant of *A. flavus*, which was sensitive to Congo red (200 μg/ml), 1 M sodium chloride, and acidic pH [38], the ΔgprK mutant of *A. fumigatus* did not exhibit altered tolerance to cell wall stressors, osmotic stressors, and pH stress (S2 Fig). However, the *A. fumigatus* ΔgprK mutant was highly sensitive to various oxidative stressors including hydrogen peroxide and menadione (MD) but not paraquat (PQ) (Fig 3B). Reductions of approximately 82 and 61% in colony radial growth for the ΔgprK mutant were observed in the presence of hydrogen peroxide and MD, respectively. To determine a potential contribution of GprK in a MAPK pathway, we analyzed mRNA levels of stress-response related genes in addition to activity of catalases and superoxide dismutases (SODs) in conidia. The expression of sakA and atfA mRNA was significantly reduced in ΔgprK mutant conidia, and accumulation of catalase and SOD mRNAs were lowered in ΔgprK strain (Fig 3C). The activities of CatA, SOD1, and SOD2 were drastically reduced in ΔgprK strain (Fig 3D), too. These results indicate that GprK is required for proper resistance of the fungus to certain oxidative stresses.

**A role of GprK in gliotoxin production and virulence**

We have shown that biosynthesis of the mycotoxin gliotoxin (GT) is in part regulated by the asexual developmental activator BrlA [39]. As the deletion of gprK resulted in impaired conidiation and lowered brlA expression, we examined whether the ΔgprK mutant would be defective in GT production. First, we performed quantitative real time PCR using total RNA of WT, mutant, and C' strains and analyzed mRNA levels of key GT biosynthetic genes. The mRNA levels of the gliM, gliP, gliT, and gliZ genes were significantly lower in the ΔgprK strain than in WT and C' strains (Fig 4A). We then assessed levels of GT itself in the three strains, and found that the ΔgprK strain produced undetectable amounts of GT (Fig 4B).

We next examined the effect of GprK on virulence using conidia invasiveness in a human cell line and survivability in the *Galleria mellonella* (wax moth) insect model. The ability of conidia to invade host cells is critical for invasive aspergillosis (IA); thus, we tested the conidia...
Multiple Roles of Aspergillus GprK
invasiveness in the type II human alveolar A549 cell line. The invasion rate of WT conidia was about 13.4% while that of conidia from the ΔgprK strain was 7.1%, a 47% reduction in invasiveness in comparison to WT (Fig 5A). To assess survivability, WT and ΔgprK strains were inoculated in wax moth larvae, and the larvae survival rate was recorded as a function of time. As shown in Fig 5B, larvae inoculated with either WT or the mutant began to die at day 3 post-inoculation, with the number of survivors continuing to decrease over the course of the experiment. In the first 5 days after infection, about 50% of insects in both strains died. There were no statistically significant differences between WT, ΔgprK, and complement strains as measured by G. mellonella survival (p value was 0.7074).
To further understand the fate of *A. fumigatus* inoculated into *G. mellonella*, infected larvae were fixed in formalin and processed for histopathology. The internal organs were disorganized in the infected larvae, and no nodules or granuloma-like structures were detected in the uninfected control larvae (S3 Fig). Fig 5C showed H&E and PAS-stained sections of infected larvae from WT and ΔgprK strains. Both strains induced similar histopathological changes in larvae, and hyphae were observed in sections from larvae infected with either strain. There was evidence of tissue damage in the infected larvae, with pigmented nodules and granulomas associated with hyphae. While most ΔgprK fungal hyphae were observed only within nodules, the WT hyphae were distributed extensively (Fig 5C).

Transcriptome analysis

To capture the genome-wide expression changes resulting from the absence of GprK, we carried out microarray analysis using ΔgprK and WT cells collected at 12 h post asexual-developmental induction. As shown in Fig 6A, the hierarchical clustering heat map based on transcriptome analysis showed that a majority of genes are down-regulated in ΔgprK strain compared to WT. Two biological replicates showed a high level of correlation (R = 0.928, Fig 6B). Of the 8,608 probes, 99 genes showed a significant differential expression (at least 1.5 fold, p-value < 0.05), of which 17 were up-regulated (higher transcript levels in ΔgprK strain than in WT strain) and 82 were down-regulated (S2 Table). Table 1 lists the genes with the highest increase in expression following loss of gprK. Using the Aspergillus Genome Database (www.aspgd.org) and previous works, we extracted all the available information on the function, localization, genetic pathway, and/or cellular process in which those genes were described or predicted to participate. The majority of the up-regulated genes were predicted to encode for conserved hypothetical proteins, with hydrophobin rodB identified as the up-regulated known gene with the maximum fold change in mutant relative to wild-type cells. Most of the down-regulated genes were related to transport (Table 2), including small oligopeptide transporter, nitrate transporter NrtB, ammonium transporter Mep2, MFS transporter, and H+/nucleoside cotransporter.

Discussion

*A. fumigatus* is an important opportunistic human pathogen that can cause high rates of mortality in immunocompromised humans [6–8]. Most other Aspergillus spp. do not cause human disease, so an understanding of how *A. fumigatus* differs from others Aspergillus spp., both in its virulence and in its ability to respond to different environments, may provide important clues that can lead to better control of and treatments for this pathogen.

Fungi have developed multiple systems to sense extracellular and intracellular signals in order to adapt to their environment. The GPCR gene family represents one of the most important and diverse sensor systems and has been found to play important roles in nutrient sensing and stress responses in many fungal species [5, 9, 12, 36, 40–44].

The 15 predicted *A. fumigatus* GPCRs fall into 9 classes as previously described [6]. GprK belongs to class VI of fungal GPCRs, which are characterized by the presence of both a 7-TM and a cytoplasmic RGS domain. A GPCR-RGS hybrid was first discovered in *Arabidopsis thaliana* (AtRGS1), which modulates plant cell proliferation via the Gpa1 Gα subunit [45]. Unlike
canonical GPCRs, AtRGS1 does not trigger the GDP-GTP exchange by a G protein. Instead, AtRGS1 interacts with the constitutively active G\(\alpha\) subunit, resulting in hydrolysis of GTP and subsequent deactivation of the G protein [45–47]. This type of GPCR has since been found in several species of filamentous fungi [6, 48–50]. Similar to other filamentous ascomycetes, A. fumigatus possess only one GprK (AFUA_4G01350). However, the exact functions for GprK have been elucidated in only two fungal species. GprK of A. flavus is involved in germination, nutrient sensing, toxin production, and pH stress response [38]. The gprK deficient mutant showed 50% of the WT germination rate and was impaired in growth on galactose and xylose. The \(\Delta gprK\) mutant was more sensitive than the wild type strain to Congo red, hyperosmotic conditions, and acidic and alkaline pHs [38]. The most-studied filamentous fungus, N. crassa, also possesses a GprK-like protein, Gpr-7. The \(\Delta gpr-7\) mutant was resistant to FK506, which inhibits the phosphatase calcineurin. However Gpr-7 did not appear to be essential for hyphal growth, asexual development, or sexual differentiation [51].

In the presence of external signals, GPCRs are sensitized and interact with heterotrimeric G proteins, resulting in the dissociation of GTP-G\(\alpha\) from the G\(\beta\gamma\) heterodimer. Activated G protein mediated signaling is transmitted via various downstream components, including cAMP-
dependent protein kinase (PKA) [1, 2, 4, 52]. In contrast to GprK of A. flavus and Gpr-7 of N. crassa, deletion of gprK caused enhanced activation of PKA, resulting in restricted asexual sporulation, reduced expression of key asexual regulators, and hyper-active conidial germination (Figs 1 & 2). These observations led to the hypothesis that a role of GprK is to negatively regulate a cAMP-dependent PKA pathway.

Table 1. Up-regulated genes in ΔgprK relative to WT.

| ID      | Locus_tag       | Product                              | Log₂FC | p-value |
|---------|-----------------|--------------------------------------|--------|---------|
| 5750049 | AFUA_8G00160    | conserved hypothetical protein        | 5.734  | 0.0148561|
| 573376  | AFUA_1G17250    | conidial hydrophobin RodB            | 4.317  | 0.0199937|
| 5736241 | AFUA_2G14320    | HHE domain protein                   | 4.130  | 0.0239697|
| 5737657 | AFUA_3G02685    | conserved hypothetical protein        | 3.460  | 0.0087669|
| 5750177 | AFUA_8G00740    | cytochrome P450, putative            | 1.757  | 0.0386637|
| 5744036 | AFUA_5G06680    | 4-aminobutyrate transaminase GatA    | 1.515  | 0.0303405|
| 5749834 | AFUA_7G06660    | conserved hypothetical protein        | 1.190  | 0.0038054|
| 5732913 | AFUA_1G15330    | conserved hypothetical protein        | 1.149  | 0.0484174|
| 5739591 | AFUA_3G12790    | conserved glutamic-acid-rich protein  | 1.096  | 0.0118515|
| 5740340 | AFUA_4G00850    | hypothetical protein                  | 1.060  | 0.0350951|
| 5748050 | AFUA_6G12090    | HET domain protein                   | 0.979  | 0.020793 |
| 5734405 | AFUA_2G04262    | C6 transcription factor, putative     | 0.863  | 0.0044365|
| 5745470 | AFUA_5G13200    | thioesterase family protein           | 0.857  | 0.0050195|
| 5748459 | AFUA_6G14050    | FAD binding domain protein            | 0.835  | 0.008598 |
| 5734686 | AFUA_2G05490    | HEAT repeat protein                   | 0.714  | 0.0364938|
| 5742321 | AFUA_4G12270    | LIM domain protein                    | 0.686  | 0.0052721|
| 5749217 | AFUA_7G03960    | thioesterase family protein           | 0.615  | 0.0336797|

doi:10.1371/journal.pone.0161312.t001

Table 2. Top 20 down-regulated genes in ΔgprK relative to WT.

| ID      | Locus_tag       | Product                              | Log₂FC | p-value |
|---------|-----------------|--------------------------------------|--------|---------|
| 5736469 | AFUA_2G15240    | small oligopeptide transporter, OPT family | -6.058 | 0.0054359|
| 5741273 | AFUA_4G07190    | ornithine carbamoyltransferase        | -5.442 | 0.0131424|
| 5746300 | AFUA_6G03140    | oligopeptide transporter, putative    | -4.836 | 0.0079149|
| 5733915 | AFUA_2G02000    | conserved hypothetical protein        | -4.321 | 6.404E-05|
| 5743196 | AFUA_5G01690    | DUF1445 domain protein                | -4.293 | 0.0386637|
| 5733419 | AFUA_1G17470    | high affinity nitrate transporter NrtB| -4.035 | 0.0242195|
| 5733093 | AFUA_1G16070    | conserved hypothetical protein        | -3.943 | 0.0178849|
| 5730314 | AFUA_1G1960     | conserved hypothetical protein        | -3.737 | 0.0060923|
| 5732457 | AFUA_1G13210    | uridine permease Fur1, putative       | -3.717 | 0.0137503|
| 5731954 | AFUA_1G10930    | ammonium transporter (Mep2), putative | -3.426 | 0.0162459|
| 5743018 | AFUA_5G00870    | conserved hypothetical protein        | -3.307 | 0.0179706|
| 5730436 | AFUA_1G02530    | MFS sugar transporter, putative       | -3.107 | 0.0101138|
| 5736911 | AFUA_2G17305    | hypothetical protein                  | -3.046 | 0.0363186|
| 5742705 | AFUA_4G14230    | MFS transporter, putative             | -2.932 | 0.0228129|
| 5738847 | AFUA_3G09390    | AMMereCR1 family protein              | -2.899 | 0.0389796|
| 5748265 | AFUA_6G13190    | H+/nucleoside cotransporter          | -2.878 | 0.0455004|
| 5741172 | AFUA_4G06620    | Glu/Leu/Phe/Val dehydrogenase, putative | -2.634 | 0.0089898|
| 5745210 | AFUA_5G12035    | conserved hypothetical protein        | -2.617 | 0.0374917|
| 5743818 | AFUA_5G05610    | cell cycle control protein Cw14/Bud31 | -2.599 | 0.0147689|
| 5735341 | AFUA_2G09860    | purine-cytosine permease             | -2.590 | 0.0298579|

doi:10.1371/journal.pone.0161312.t002
While a nutrient-sensing GPCR has not been identified in the *A. fumigatus*, GprK may be involved in carbon source sensing. Indeed, a majority of down-regulated genes in the ΔgprK mutant were transport-related genes (Table 2). GprK also appears to be involved in response to oxidative stress. The gprK deletion mutant was hypersensitive to hydrogen peroxide and menadione (Fig 3) but not to cell wall stressors, hyperosmotic conditions, and pH stresses (S2 Fig). Activated G-protein mediated signaling is transmitted through mitogen-activated protein kinase (MAPK) pathways [1, 2, 4]. Stress MAPK (SakA) is involved in stress signal transduction and is required for spore stress resistance and survival [53, 54]. It interacts with ATF/CREB transcription factor AtfA, and the SakA-AtfA interaction regulates gene expression during oxidative stress in *A. nidulans* [54]. It has been demonstrated that accumulation of spore-specific *catA* mRNA was dependent on SakA and AtfA [54], and *CatA* activity in ΔsakA conidia was lower than in WT conidia [53]. The *sod1* and *sod2* of *A. fumigatus* were highly expressed in conidia, and Δsod1 and Δsod2 mutants showed hyper-sensitivity to menadione, which produces intracellular superoxide radicals [55]. The expression of *sakA* and *atfA* mRNA was significantly reduced in the ΔgprK mutant conidia and *CatA*, SOD1, and SOD2 activities were also drastically reduced in ΔgprK strain (Fig 3). Based on our results, we propose that GprK positively regulates the SakA and AtfA, and the SakA-AtfA controlled expression of conidia-specific catalase and SODs.

The GT biosynthetic gene cluster is composed of numerous genes. The *gliM* gene is predicted to encode an *o*-methyltransferase [56], and GliP catalyzes the first steps of GT biosynthesis [57]. The *gliT* gene encodes an oxidoreductase of the GT biosynthetic cluster [58, 59] and GliZ, a Zn2Cys6 transcription factor, is responsible for general GT induction and regulation [60, 61]. Expression of GT biosynthetic genes and GT production in ΔgprK strain was severely reduced compared to WT and C' strain (Fig 4), suggesting that GprK regulates GT synthesis positively, likely by activating the asexual developmental regulator *brlA*. GT is an important virulence determinant of *A. fumigatus* and the ΔgliP strain was significantly less virulent and deletion of *gliP* abrogated GT production [62].

The ability of conidia to invade host cells is also a critical virulence determinant. Toxins that are secreted by conidia, such as GT, can affect ciliary movement of the host’s bronchial epithelial cells and increase the chance of colonization [63]. The invasion rate of conidia for the ΔgprK strain was significantly reduced (about 47% of WT), suggesting that GprK plays a role in virulence. A previous study also found a positive correlation between the level of GT production and pathogenicity level in greater wax moths [64]. Larvae infected with the low GT-producing strains exhibited reduced mortality, while the high mortality of larvae infected with the high GT producing strain suggested that GT production was a significant contributor to the pathogenicity of *A. fumigatus* in *G. mellonella*. Survival analysis of *G. mellonella* larvae challenged with the ΔgprK mutant and WT have revealed that the mortality level of ΔgprK strain was similar to that of WT strain (Fig 5B). Microscopically, we observed the presence of granuloma-like structures in infected larval tissue, while no nodules or granuloma-like structures were detected in the uninfected control larvae (S3 Fig). While most of fungal hyphae of the ΔgprK mutant were observed only within nodules of infected larvae, the hyphae of the WT were distributed extensively (Fig 5C).

It was revealed that the hydrophobin *rodB* was up-regulated in the ΔgprK mutant with the maximum fold change in our microarray analysis (Table 1). RodB, which is specific to *A. fumigatus*, is involved in building the conidial outer cell wall, but does not protect conidia against killing by lung alveolar macrophages [65]. Most of the genes down-regulated in the ΔgprK mutant were related to transport, such as the small oligopeptide transporter, nitrate transporter NrtB, ammonium transporter Mep2, MFS transporter, and H+/nucleoside cotransporter (Table 2). *A. nidulans* possesses two nitrate transporters, NrtA and NrtB [66, 67], and nitrate...
transport has been shown to be proton-dependent [68]. The NrtA and NrtB nitrate transporters are paralogous members of the major facilitator superfamily (MFS) and the NrtB transporter may be more effective at scavenging nitrate from low external concentrations [66]. Ammonium transporter Mep2 is a transporter for ammonium to use as a nitrogen source and under ammonium limitation, Mep2 acts as an ammonium sensor, generating a signal that leads to pseudohyphal growth in *S. cerevisiae* [69]. These results suggest that the ΔgprK mutant fails to sense external nutrients effectively, which may be associated with down-regulation of a majority of transporters.

**Supporting Information**

S1 Fig. Fifteen predicted GPCRs in *A. fumigatus* and a phylogenetic tree of GprK-like proteins in filamentous fungi. (A) Predicted *A. fumigatus* GPCRs are presented schematically using SMART (http://smart.embl-heidelberg.de) with the number of TMs shown in parenthesis. Small pink rectangles are indicated low complexity regions. (B) The phylogenetic tree is constructed based on the matrix of pair-wise distances between sequences. NFIA 043940: hypothetical protein of *N. fischeri* NRRL 181, ACLA 067510: conserved hypothetical protein of *A. clavatus* NRRL 1, ANID 07795.1: conserved hypothetical protein of *A. nidulans* FGSC A4, ATEG 08180.1: conserved hypothetical protein of *A. terreus* NIH2624, AO090103000244: hypothetical protein of *A. oryzae* RIB40, AFL2G 12145.2: conserved hypothetical protein of *A. flavus* NRRL3357, ASPNIDRAFT 131536: hypothetical protein of *A. niger* ATCC 1015. (C) Alignment of the GprK homologs of *A. fumigatus* (Afu4g01350), *A. niger* (An04g07760), and *A. nidulans* (AN7795). The identical amino acids are marked by shades. ClustalW (http://align.genome.jp/) was used for the alignment.

(PPTX)

S2 Fig. Sensitivity of mutant lacking gprK towards cell wall stress, osmotic stress, and different pH levels. About 1×10⁶ spores of each strain was spotted on to YG agar plates supplemented with indicated amount of Congo red (CR), calcofluor white (CFW), caspofungin (CAS), sodium chloride, or sorbitol and incubated at 37°C for 48 h. Additional plates contained media buffered to three different pHs before autoclaving. Statistical significance was determined by a Student’s t-test, with *p < 0.05 and **p < 0.01.

(PPTX)

S3 Fig. Histological analysis of larval tissues infected by *A. fumigatus*. (A) Uninfected control larvae and (B) infected larvae with WT. Note that the internal organs were not clear in the infected larvae and no nodules or granuloma-like structures were detected in the uninfected control larvae. Arrows indicate nodules or granulomas-like structures. c: cuticle, a: adipose tissue, g: gastrointestinal tract.

(PPTX)

S1 Table. Oligonucleotides used in this study.

(DOC)

S2 Table. Significantly differentially expressed genes in ΔgprK relative to WT (≥1.5 fold, p-value < 0.05).

(DOC)

**Acknowledgments**

We thank Dr. Wendy Bedale of Food Research Institute at UW-Madison for critically editing our manuscript, and our lab members for helpful discussions.
Author Contributions

Conceptualization: JHY KSS.
Formal analysis: MGJ SSK JHY KSS.
Funding acquisition: JHY KSS.
Investigation: MGJ SSK KSS.
Methodology: MGJ JHY KSS.
Project administration: KSS.
Resources: JHY KSS.
Supervision: JHY KSS.
Visualization: MGJ KSS.
Writing – original draft: MGJ JHY KSS.
Writing – review & editing: JHY KSS.

References

1. Morris AJ, Malbon CC. Physiological regulation of G protein-linked signaling. Physiol Rev. 1999; 79(4):1373–430. PMID:10508237.
2. McCudden CR, Hains MD, Kimple RJ, Siderovski DP, Willard FS. G-protein signaling: back to the future. Cell Mol Life Sci. 2005; 62(5):551–77. doi: 10.1007/s00018-004-4462-3 PMID:15747061; PubMed Central PMCID: PMCPMC2794341.
3. Neves SR, Ram PT, Iyengar R. G protein pathways. Science. 2002; 296(5573):1636–9. doi: 10.1126/science.1071550 PMID:12040175.
4. Yu JH. Heterotrimeric G protein signaling and RGSs in Aspergillus nidulans. J Microbiol. 2006; 44(2):145–54. PMID:16728950.
5. Xue C, Hsueh YP, Heitman J. Magnificent seven: roles of G protein-coupled receptors in extracellular sensing in fungi. FEMS Microbiol Rev. 2008; 32(6):1010–32. doi: 10.1111/j.1574-6976.2008.00131.x PMID:18911658; PubMed Central PMCID: PMCPMC2998294.
6. Lafon A, Han KH, Seo JA, Yu JH, d’Enfert C. G-protein and cAMP-mediated signaling in aspergilli: a genomic perspective. Fungal Genet Biol. 2006; 43(7):490–502. doi: 10.1016/j.fgb.2006.02.001 PMID:16546420.
7. Cockrill BA, Hales CA. Allergic bronchopulmonary aspergillosis. Annu Rev Med. 1999; 50:303–16. doi: 10.1146/annurev.med.50.1.303 PMID:10073280.
8. Dagenais TR, Keller NP. Pathogenesis of Aspergillus fumigatus in Invasive Aspergillosis. Clin Microbiol Rev. 2009; 22(3):447–65. doi: 10.1128/CMR.00055-08 PMID:19597008; PubMed Central PMCID: PMCPMC2708386.
9. Han KH, Seo JA, Yu JH. A putative G protein-coupled receptor negatively controls sexual development in Aspergillus nidulans. Mol Microbiol. 2004; 51(5):1333–45. doi: 10.1111/j.1365-2958.2003.03940.x PMID:14982628.
10. Galagan JE, Calvo SE, Cuomo C, Ma LJ, Wortman JR, Batzoglou S, et al. Sequencing of Aspergillus nidulans and comparative analysis with A. fumigatus and A. oryzae. Nature. 2005; 438(7071):1105–15. doi: 10.1038/nature04341 PMID:16372000.
11. Nierman WC, Pain A, Anderson MJ, Wortman JR, Kim HS, Arroyo J, et al. Genomic sequence of the pathogenic and allergenic filamentous fungus Aspergillus fumigatus. Nature. 2005; 438(7071):1151–6. doi: 10.1038/nature04332 PMID:16372009.
12. Xue Y, Battle M, Hirsch JP. GPR1 encodes a putative G protein-coupled receptor that associates with the Gpa2p Gα subunit and functions in a Ras-independent pathway. EMBO J. 1998; 17(7):1996–2007. doi: 10.1093/emboj/17.7.1996 PMID:9524122; PubMed Central PMCID:PMCPMC1170545.
13. Kraakman L, Lemaire K, Ma P, Teunissen AW, Donaton MC, Van Diéck P, et al. A Saccharomyces cerevisiae G-protein coupled receptor, Gpr1, is specifically required for glucose activation of the CAM pathway during the transition to growth on glucose. Mol Microbiol. 1999; 32(5):1002–12. PMID:10961302.
Bok JW, Keller NP. LaeA, a regulator of secondary metabolism in Wayne LG, Diaz GA. A double staining method for differentiating between two classes of mycobacterial 30. Beauchamp C, Fridovich I. Superoxide dismutase: improved assays and an assay applicable to acryl-31. Ni M, Rierson S, Seo JA, Yu JH. The 32. Fuchs BB, O’Brien E, Khoury JB, Mylonakis E. Methods for using Galleria mellonella as a model host to study fungal pathogenesis. Virulence. 2010; 1(6):475–82. PMID: 21178491.

Fillinger S, Chaveroche MK, Shimizu K, Keller N, d’Enfert C. cAMP and ras signalling independently control spore germination in the filamentous fungus Aspergillus nidulans. Mol Microbiol. 2002; 44 (4):1001–16. PMID: 12046590.
34. Han KH, Seo JA, Yu JH. Regulators of G-protein signalling in Aspergillus nidulans: RgsA downregulates stress response and stimulates asexual sporulation through attenuation of GαB (Go) signalling. Mol Microbiol. 2004; 53(2):529–40. doi: 10.1111/j.1365-2958.2004.04163.x PMID: 15228532.

35. Lafon A, Seo JA, Han KH, Yu JH, d’Enfert C. The heterotrimeric G-protein GanB(α)-SfaD(β)-GpgA(γ) is a carbon source sensor involved in early cAMP-dependent germination in Aspergillus nidulans. Genetics. 2005; 171(1):71–80. doi: 10.1534/genetics.105.040584 PMID: 15944355; PubMed Central PMCID: PMCPMC1456537.

36. Lemaire K, Van de Velde S, Van Dijck P, Thevelein JM. Glucose and sucrose act as agonist and mannoside as antagonist ligands of the G protein-coupled receptor Gpr1 in the yeast Saccharomyces cerevisiae. Mol Cell. 2004; 16(2):293–9. doi: 10.1016/j.molcel.2004.04.004 PMID: 15494315.

37. Li L, Borkovich KA. GPR-4 is a predicted G-protein-coupled receptor required for carbon source-dependent asexual growth and development in Neurospora crassa. Eukaryot Cell. 2006; 5(8):1287–300. doi: 10.1128/EC.00109-06 PMID: 16896213; PubMed Central PMCID: PMCPMC1539153.

38. Affeldt KJ, Carrig J, Amare M, Keller NP. Global survey of canonical Aspergillus flavus G protein-coupled receptors. MBio. 2014; 5(5):e01501–14. doi: 10.1128/mBio.01501-14 PMID: 25316696; PubMed Central PMCID: PMCPMC4205791.

39. Shin KS, Kim YH, Yu JH. Proteomic analyses reveal the key roles of BrlA and AbaA in biogenesis of gliotoxin in Aspergillus fumigatus. Biochem Biophys Res Commun. 2015; 463(3):428–33. doi: 10.1016/j.bbrc.2015.05.090 PMID: 26032501.

40. Miwa T, Takagi Y, Shinozaki M, Yun CW, Schell WA, Perfect JR, et al. Gpr1, a putative G-protein-coupled receptor, regulates morphogenesis and hypha formation in the pathogenic fungus Candida albicans. Eukaryot Cell. 2004; 3(4):919–31. doi: 10.1128/EC.3.4.919–931.2004 PMID: 15302825; PubMed Central PMCID: PMCPMC5008777.

41. Bordwell L. A walk-through of the yeast mating pheromone response pathway. Peptides. 2004; 25(9):1465–76. doi: 10.1016/j.peptides.2003.10.022 PMID: 15374648.

42. Lorenz MC, Pan X, Harashima T, Cardenas ME, Xue Y, Hirsch JP, et al. The G protein-coupled receptor gpr1 is a nutrient sensor that regulates pseudohyphal differentiation in Saccharomyces cerevisiae. Genetics. 2000; 154(2):609–22. PMID: 10665215; PubMed Central PMCID: PMCPMC1460933.

43. Maidan MM, Thevelein JM, Van Dijck P. Carbon source induced yeast-to-hypha transition in Candida albicans is dependent on the presence of amino acids and on the G-protein-coupled receptor Gpr1. Biochim Soc Trans. 2005; 33(Pt 1):291–3. doi: 10.1042/BST0330291 PMID: 15667329.

44. Maidan MM, De Rop L, Semeels J, Exler S, Rupp S, Touru M, et al. The G protein-coupled receptor Gpr1 and the Gα protein Gpa2 act through the cAMP-protein kinase A pathway to induce morphogenesis in Candida albicans. Mol Biol Cell. 2005; 16(4):1971–86. doi: 10.1091/mbc.E04-09-0780 PMID: 15673611; PubMed Central PMCID: PMCPMC1073676.

45. Chen JG, Willard FS, Huang J, Liang J, Chasse SA, Jones AM, et al. A seven-transmembrane RGS protein that modulates plant cell proliferation. Science. 2003; 301(5640):1728–31. doi: 10.1126/science.1087790 PMID: 14500984.

46. Chen JG, Jones AM. AirG51 function in Arabidopsis thaliana. Methods Enzymol. 2004; 389:338–50. doi: 10.1016/S0076-6879(04)80920-7 PMID: 15313575.

47. Johnston CA, Taylor JP, Gao Y, Kimple AJ, Grigston JC, Chen JG, et al. GTPase acceleration as the rate-limiting step in Arabidopsis G protein-coupled sugar signaling. Proc Natl Acad Sci U S A. 2007; 104(44):17317–22. doi: 10.1073/pnas.0704751104 PMID: 17951432; PubMed Central PMCID: PMCPMC2077254.

48. Brunner K, Omann M, Pucher ME, Delic M, Lehner SM, Domannich P, et al. Trichoderma G protein-coupled receptors: functional characterisation of a cAMP receptor-like protein from Trichoderma atroviride. Curr Genet. 2008; 54(6):283–99. doi: 10.1007/s00294-008-0217-7 PMID: 19836726; PubMed Central PMCID: PMCPMC2855676.

49. Li L, Wright SJ, Krystofova S, Park G, Borkovich KA. Heterotrimeric G protein signalling in filamentous fungi. Annu Rev Microbiol. 2007; 61:423–52. doi: 10.1146/annurev.micro.61.080706.093432 PMID: 17506873.

50. Gruber S, Omann M, Zeilinger S. Comparative analysis of the repertoire of G protein-coupled receptors of three species of the fungal genus Trichoderma. BMC Microbiol. 2013; 13:108. doi: 10.1186/1471-2180-13-108 PMID: 23679152; PubMed Central PMCID: PMCPMC3664084.

51. Cabrera IE, Pacentine IV, Lim A, Guerrero N, Krystofova S, Li L, et al. Global Analysis of Predicted G Protein-Coupled Receptor Genes in the Filamentous Fungus, Neurospora crassa. G3 (Bethesda). 2015; 5(12):2729–43. doi: 10.1534/g3.115.020974 PMID: 26464358; PubMed Central PMCID: PMCPMC4683645.
