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RESEARCH ARTICLE

Genetic relationships between the RACK1 homolog cpc-2 and heterotrimeric G protein subunit genes in *Neurospora crassa*

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Abstract

Receptor for Activated C Kinase-1 (RACK1) is a multifunctional eukaryotic scaffolding protein with a seven WD repeat structure. Among their many cellular roles, RACK1 homologs have been shown to serve as alternative Gβ subunits during heterotrimeric G protein signaling in many systems. We investigated genetic interactions between the RACK1 homolog cpc-2, the previously characterized Gβ subunit gnb-1 and other G protein signaling components in the multicellular filamentous fungus *Neurospora crassa*. Results from cell fractionation studies and from fluorescent microscopy of a strain expressing a CPC-2-GFP fusion protein revealed that CPC-2 is a cytoplasmic protein. Genetic epistasis experiments between cpc-2, the three Gα genes (gna-1, gna-2 and gna-3) and gnb-1 demonstrated that cpc-2 is epistatic to gna-2 with regards to basal hyphae growth rate and aerial hyphae height, while deletion of cpc-2 mitigates the increased macroconidiation on solid medium observed in Δgnb-1 mutants. Δcpc-2 mutants inappropriately produce conidiophores during growth in submerged culture and mutational activation of gna-3 alleviates this defect. Δcpc-2 mutants are female-sterile and fertility could not be restored by mutational activation of any of the three Gα genes. With the exception of macroconidiation on solid medium, double mutants lacking cpc-2 and gnb-1 exhibited more severe defects for all phenotypic traits, supporting a largely synergistic relationship between GNB-1 and CPC-2 in *N. crassa*.

Introduction

Heterotrimeric G protein signaling cascades consist of seven-helix transmembrane G Protein-Coupled Receptors (GPCRs) and the three G protein subunits—Gα, Gβ and Gγ [1–3]. In the inactive state, the Gαβγ heterotrimer is associated with the GPCR. Ligand stimulation causes
exchange of GDP for GTP on the Gα, leading to dissociation of Gα-GTP from the Gβγ heterodimer. The Gα-GTP and the Gβγ dimer can then regulate downstream effectors, leading to changes in cellular physiology [3]. The Gα-GTP has native GTPase activity that causes release of the inorganic phosphate from the GTP. The Gα-GDP then reassociates with the Gβ subunit and GPCR, leading to signal termination and completion of the cycle.

Neurospora crassa is a multicellular ascomycete fungus that has emerged as a model system to study G protein signaling, and comparisons with N. crassa have driven discoveries in pathogenic fungi and higher eukaryotes [4, 5]. In N. crassa, there are 43 predicted GPCRs, three Gα subunits (GNA-1, GNA-2 and GNA-3), one characterized Gβ subunit (GNB-1) and one Gγ subunit (GNG-1) [6, 7]. Major processes such as hyphal growth, macroconidiation, conidial germination, mating, nutrient sensing and temperature and oxidative stress resistance are regulated by G protein signaling pathways in N. crassa [8–14].

Receptor for Activated C Kinase-1 (RACK1) is a major scaffolding protein in many eukaryotic systems. Similar to G protein β subunits, RACK1 has a seven WD repeat structure, and is one of the best-studied proteins in the WD-repeat family [15]. Initially identified as a protein that binds to the active conformation of protein kinase C (PKC) βII, RACK1 is now known to be multifunctional [16, 17]. For example, RACK1 allows cross talk between the PKC and Mitogen Activated Kinase (MAP) pathways by acting as a scaffold for the Jun N-terminal Kinase (JNK) upon stimulation, leading to PKC-mediated phosphorylation and activation of JNK [18]. It has been observed that RACK1 binds to the Gβγ dimer in HEK293 cells and also regulates a subset of its functions, including promoting its dislocation from the cytosol to the membrane [19]. Additionally, RACK1 is known to associate with the 40S subunit of the ribosome, near the mRNA exit channel [20]. Due to its conformation when bound to the ribosome, RACK1 is believed to serve as an adaptor, bringing together proteins at the ribosome during translation [reviewed in [15]].

Homologs of RACK1 have been implicated as alternative Gβ subunits in the fungal kingdom, through direct interaction with Gα subunits [21, 22]. In Saccharomyces cerevisiae, Asc1p functions as a Guanine nucleotide Dissociation Inhibitor (GDI) for the Gα Gpa2, and is involved in regulating glucose responsiveness through its binding to adenylyl cyclase (Cyr1) [23]. gib2, an essential gene in Cryptococcus neoformans, encodes a protein that binds to the Gα Gpa1 and two Gγ subunits, Gpg1 and Gpg2. It also associates with Smg1, a downstream target of cAMP signaling, and to the protein kinase C homolog Pkc1 [24]. In Magnaporthe oryzae, the RACK1 ortholog MoMip11 interacts with the Gα protein MoMagA and the Regulator of G protein Signaling (RGS) protein MoRgs7 to regulate pathogenicity [25, 26].

Additional RACK1 orthologs have been shown to regulate various aspects of growth and development in several fungal systems, but without demonstration of a physical interaction with heterotrimeric Gα proteins. S. pombe Cpc2 plays a role in cell cycle regulation and stress responses through ribosomal association [27] and translational control of the stress response transcriptional factor Atf1 [27]. RACK1 orthologs from Aspergillus nidulans and Aspergillus fumigatus have been demonstrated to regulate sexual differentiation and asexual growth and development, respectively [28, 29]. In Ustilago maydis, Rak1 is essential for the transcription of rop1, which is a direct positive regulator of the pheromone response factor (prf), making it essential for mating [30]. Strains lacking RAK1 also have attenuated filamentation and virulence, and abnormal cell morphology [30].

The N. crassa RACK1 homolog CPC-2 was the first reported RACK1 protein in fungi, initially identified as a component of the general amino acid regulation network [31]. In N. crassa, starvation for a single amino acid leads to an overall derepression of all amino acid biosynthetic genes at the level of transcription [32]. Loss of the cpc-2 gene blocks derepression of amino acid biosynthetic genes during amino acid limiting conditions [31]. Under
non-starved conditions, loss of the cpc-2 gene decreases growth by 50% [33]. During the sexual cycle, the \( \Delta \)cpc-2 mutant lacks protoperithecia, and is female-sterile [33]. Other components of this cross pathway control network are cpc-1, homologous to GCN4 [34], and cpc-3, the \( N. \) crassa equivalent of GCN2 [35]. Analysis of \( \Delta \)cpc-2 \( \Delta \)cpc-3 and \( \Delta \)cpc-2 \( \Delta \)cpc-1 double mutants showed that they possessed \( \Delta \)cpc-2 phenotypes, such as reduced growth and female sterility. These findings suggested that cpc-2 has broader functions operating outside of amino acid control [35].

To-date, no one has explored a possible function for CPC-2 in G protein signaling in \( N. \) crassa. In this study, we use strains carrying single and double gene deletions or expressing constitutively activated G\( \alpha \) alleles to analyze genetic epistasis between components of the G protein pathway and the cpc-2 gene. We produce a polyclonal antibody against CPC-2 and use western analysis to determine protein levels in the mutants lacking the other G protein subunits. Our results reveal that \( N. \) crassa mutants lacking both predicted G\( \beta \) subunits are viable, but possess major defects in growth and development. We also provide evidence for G protein dependent and independent functions for CPC-2 in \( N. \) crassa.

Materials and methods

Strains and media

\( N. \) crassa strains were either obtained from the Fungal Genetics Stock Center (FGSC; Kansas State University, Manhattan, KS) [36] or created during this work (Table 1). Strains that are not deposited in the FGSC collection are available upon request. Strains were cultured in Vogel’s minimal medium (VM) [37] to propagate vegetative hyphae or asexual spores (macroconidia; conidia). Synthetic Crossing Medium (SCM) plates containing 1% agar were used to induce development of female sexual reproductive structures [38]. Sorbose-containing medium (FGS) was used to facilitate colony formation on plates [39]. Media was supplemented with 100 \( \mu \)g/ml of histidine, 10 \( \mu \)g/ml pantothenate, 200 \( \mu \)g/ml hygromycin (Calbiochem, San Diego, CA), 200 \( \mu \)g/ml nourseothricin (Werner BioAgents, Germany) or 400 \( \mu \)g/ml phosphinothricin (purified from Finale, Farnam Companies, Inc., Phoenix, AZ), where indicated. Conidia were propagated in VM agar flasks as described previously [39]. Liquid cultures were brought to a concentration of 1x10^6 conidia/ml and incubated with shaking at 200 RPM at 30˚C for 16 hr. Escherichia coli strain DH5\( \alpha \) was used to maintain all plasmids.

Phylogenetic analysis

Protein sequences orthologous to \( N. \) crassa CPC-2 (NCU05810) and GNB-1 (NCU00440) from 18 fungal species chosen to represent a diversity of fungi [40] were obtained from the FungiDB database (fungidb.org) [41]. Sequences for the G\( \beta \) and RACK1 proteins from the plant Arabidopsis thaliana were retrieved from the National Center for Biotechnology Information (NCBI). The “One-Click Workflow” tool at NGPhylogeny.fr [42] was implemented for the phylogenetic analysis. This pipeline uses FASTA files to generate a multiple alignment using MAFFT (Multiple Alignment using Fast Fourier Transform) [43]. Alignments were inspected and proteins from the 18 species resulted in good alignments for both the G\( \beta \) and RACK1. The MAFFT alignments were curated using BMGE (Block Mapping and Gathering with Entropy) [44] and FastME (Fast Minimum Evolution) [45] was used to produce the tree file. FastME uses distance algorithms to infer phylogenies. The final trees were drawn using tools at the Interactive Tree of Life (iTOL; itol.embl.de) [46]. Species and gene accession numbers are in the legend to Fig 1.
Table 1. *Neurospora crassa* strains used in this study.

| Strain name               | Relevant genotype                          | Comments                                                                 | Source or Reference |
|---------------------------|--------------------------------------------|--------------------------------------------------------------------------|---------------------|
| 74-OR23-1VA               | Wild type, mat A                           |                                                                          | FGSC1 2489          |
| ORS-SL6a                  | Wild type, mat a                           |                                                                          | FGSC2400            |
| 74A-OR23-1A               | Wild type, mat A                           |                                                                          | FGSC987             |
| a<sup>m1</sup>            |ocy-1, ad3B, a<sup>m1</sup>                |                                                                          | FGSC4564            |
| Y234M723                  | his-3, mat A                               |                                                                          | FGSC6103            |
| his-3A#14                 | his-3, mat a                               |                                                                          | Ref. [61]           |
| 3B10                      | Δgna::hph(Δ) mat a                         |                                                                          | Ref. [81]           |
| Δgna2-2477                | Δgna::hph(Δ) mat a                         |                                                                          | FGSC12377           |
| Δgna2-2476                | Δgna::hph(Δ) mat A                         |                                                                          | FGSC12376           |
| 31c2                      | Δgna3::hph(Δ), mat A                       |                                                                          | Ref. [14]           |
| 42-8-3                    | Δgna1::hph(Δ), mat A                       |                                                                          | Ref. [64]           |
| Δcpc2Het                  | Δcpc2::hph<sup>+</sup>, Δmus-51::bar<sup>+</sup>, mat A (heterokaryon) |                                                                          | FGSC12365           |
| Δcpc2#1                   | Δcpc2::hph<sup>+</sup>, Δmus-51::bar<sup>+</sup>, mat a | Progeny from cross of Δcpc2Het to 74-OR23-1VA                         | This Study          |
| Δcpc2#6                   | Δcpc2::hph<sup>+</sup>, Δmus-51::bar<sup>+</sup>, mat a | Progeny from cross of Δcpc2Het to 74-OR23-1VA                         | This Study          |
| Δcpc2#11                  | Δcpc2::hph<sup>+</sup>, mat A              |                                                                          | Progeny from cross of Δcpc2Het to 74-OR23-1VA                         | This Study          |
| Δcpc2his3A                | Δcpc2::hph<sup>+</sup>, his-3, mat A       |                                                                          | Progeny from cross of Δcpc2Het to Y234M723                             | This Study          |
| Δcpc2<sup>a<sup>m1</sup>  | Δcpc2::hph<sup>+</sup>, his-3, mat A + a<sup>m1</sup>, cyh-1, ad3B, mat A (heterokaryon) | Heterokaryon of Δcpc2his3A and a<sup>m1</sup>                            | This Study          |
| C2G1#44                   | Δcpc2::hph<sup>+</sup>, his-3::gna-<sup>3</sup>-<sup>gna-3</sup><sup>2004</sup>, mat A | Δcpc2his3A purified transformant                                        | This Study          |
| C2G2#4                    | Δcpc2::hph<sup>+</sup>, his-3::gna-<sup>3</sup>-<sup>gna-3</sup><sup>2004</sup>, mat A | Δcpc2his3A purified transformant                                        | This Study          |
| C2G3#1<sup>–</sup>        | Δcpc2::hph<sup>+</sup>, his-3::gna-<sup>3</sup>-<sup>gna-3</sup><sup>2004</sup>, mat A | Δcpc2his3A purified transformant                                        | This Study          |
| C2G1#39                   | Δcpc2::hph<sup>+</sup>, Δgna1::hph<sup>+</sup>, mat a | Progeny from cross of Δcpc2<sup>a<sup>m1</sup></sup> to 3b10           | This Study          |
| C2G2#37                   | Δcpc2::hph<sup>+</sup>, Δgna2::hph<sup>+</sup>, mat a | Progeny from cross of Δcpc2<sup>a<sup>m1</sup></sup> to Δgna2-2477           | This Study          |
| C2G3#1<sup>–</sup>        | Δgna3::hph<sup>+</sup>, Δcpc2::hph<sup>+</sup>, mat a | Progeny from cross of Δgna3 to Δcpc2<sup>a</sup>                       | This Study          |
| C2B1#2-1-1                | Δgna2::nat<sup>+</sup>, Δcpc2::hph<sup>+</sup>, mus-51::bar<sup>+</sup>, mat a | Δcpc2<sup>a</sup> purified transformant                                | This Study          |
| 51-4                      | Δadi:Δnat<sup>+</sup>, Δmus-51::nat<sup>+</sup>, mat a |                                                                          | This Study          |
| CPC-2-GFP-9               | Δpan-2::pcg1-1::pcg2-2-V5-GFP::bar<sup>+</sup>, mat a | 51-4-1 transformant                                                    | This Study          |
| CPC-2-GFP-9-10            | Δpan-2::pcg1-1::pcg2-2-V5-GFP::bar<sup>+</sup>, mat a | Progeny of CPC-2-GFP-9 crossed to 74-OR23-1VA                             | This Study          |
| pccg1-1_GFP               | Δpan-2::pcg1-1::V5-GFP::bar<sup>+</sup>, mat a | Empty vector control for CPC-2-GFP-9-10                                    | Ref. [38]           |
| CPC-2-GFP-13              | Δpan-2::pcg1-1::pcg2-2-V5-GFP::bar<sup>+</sup>, mat a | 51-4-1 transformant                                                    | This Study          |
| CPC-2-GFP-13.2            | Δpan-2::pcg1-1::pcg2-2-V5-GFP::bar<sup>+</sup>, mat a | Progeny of CPC-2-GFP-13 crossed to 74-OR23-1VA                             | This Study          |

<sup>*FGSC: Fungal Genetics Stock Center, Kansas State University, Manhattan, KS [36]</sup><br><br>https://doi.org/10.1371/journal.pone.0223334.1001

**N. crassa** strain construction

The Δcpc-2::hph<sup>R</sup> knockout mutant was deposited at the FGSC as a heterokaryon (FGSC13695). Homokaryotic mutants were obtained from the heterokaryon after a sexual cross to wild type strain 74-OR23-1VA and plating ascospores on medium containing hygromycin. Progeny were checked using diagnostic PCR [47] with cpc-2 (Primer 1 or Primer 2) and hph (Primer 13 or Primer 14) primers (Table 2; S1 Fig), and then spot-tested on phosphinothricin to check for the presence of the mus-51 mutation, which is marked with bar [48, 49].

Double mutants Δcpc-2, Δgna-1; Δcpc-2, Δgna-2 and Δcpc-2, Δgna-3 were made using genetic crosses between single mutants [39] (Table 1). In cases where both single mutants in the cross were female-sterile (Δcpc-2 and Δgna-1), the strain used as the female was a heterokaryon with the a<sup>m1</sup> helper strain [50]. The presence of the mutations in the progeny was verified by diagnostic PCR using pairs of gene-specific and hph cassette-specific primers (Primers 1–14 in Table 2; S1 Fig).
Fig 1. Phylogenetic analysis of Gβ and RACK1 proteins from 10 fungal species. Amino acid sequences were obtained from FungiDB or NCBI and phylogenetic analysis conducted using the “One-Click Workflow” tool at NGPhylogeny.fr. The final trees were drawn using tools atitol.embl.de (see Materials and methods for details). A. Gβ proteins. Organisms and protein names/accession numbers for the Gβ orthologs are Neurospora crassa NcGNB-1/NCU00440; Sordaria macrospora SmGbta/SMAC01876; Fusarium graminearum GzGBP1/FRGAMPH101G14499; Magnaporthe oryzae MoMgb1/MGG05201; Aspergillus nidulans AnSfaD/AN0081; Ustilago maydis UmBpp1/UMAG00703; Cryptococcus neoformans CnGpb1/CNAG01262; Candida albicans CaSte4/C204210W A; Schizosaccharomyces pombe SpGit5/SPBC32H8.07 and Saccharomyces cerevisiae ScSte4/YOR212W; Batrachochytrium dendrobatidis/BDEG_08231; Botrytis cinerea/Bcin08g01420; Puccinia graminis f. sp. tritici/PGTG_03572; Sporisorium reilianum/sr11991; Spizellomyces
Repeated attempts to generate a Δcpc-2 Δgnb-1 double mutant through a sexual cross were unsuccessful. Therefore, the double mutant was created by electroporation of the Δcpc2#6 strain using a knockout cassette for gnb-1, marked with nourseothricin resistance (nat^R) [51]. The Δgnb-1 knockout cassette was created using yeast recombinational cloning in vector punctatus/SPPG_02467; Phycomyces blakesleeanus/PHYBL_104565; Phycomyces blakesleeanus/PHYBL_139838; Phycomyces blakesleeanus/PHYBL_14376; Phycomyces blakesleeanus/PHYBL_153895; Phycomyces blakesleeanus/PHYBL_79980; Mucor circinelloides f. lusitanicus/QYA_112430; Mucor circinelloides f. lusitanicus/QYA_167321; Mucor circinelloides f. lusitanicus/QYA_177085; Rhizophagus irregularis/GLOIN_2v1532112; Arabidopsis thaliana/ABR138682.1

**B. RACK1 proteins.** Organisms and protein names or accession numbers for the RACK1 orthologs are Neurospora crassa NcCPC-2/NcRACK1/NCU05810; Sordaria macrospora SmRACK1/SMAC07639; Fusarium graminearum FgRACK1/FGRAMPH101G06721; Magnaporthe oryzae MoRACK1/MMG04719; Aspergillus nidulans AnCpcB/AN4163; Ustilago maydis UmRACK1/UMAG015146; Cryptococcus neoformans CnGib2/CNAG05465; Candida albicans CaAsc1/CT01250WA; Schizosaccharomyces pombe SpCpc2/SPAC6B12.15; Saccharomyces cerevisiae ScAsc1/YMR116C; Batrachochytrium dendrobatidis/BDG_04723; Botrytis cinerea/Bcin14g03010; Sporisorium reilianum/sr10817; Puccinia graminis f. sp. tritici/PTG1_14970; Phycomyces blakesleeanus/PHYBL_133989; Phycomyces blakesleeanus/PHYBL_160317; Mucor circinelloides f. lusitanicus/QYA_155892; Mucor circinelloides f. lusitanicus/QYA_156660; Rhizophagus irregularis/GLOIN_2v153218; Arabidopsis thaliana/AT OAP14939.1.

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Table 2. Oligonucleotides used in this study.

| Primer # | Primer name          | Sequence 5’ to 3’ |
|----------|----------------------|-------------------|
| 1        | CPC2FOR DIAG         | AGCAGGGCCGGGTTGAGATT |
| 2        | CPC2REV DIAG         | CGAAGGTCACCCCTAACAGCC |
| 3        | GNA1FOR DIAG-1       | CTTTGAGAGTGGCGGGTTGG |
| 4        | GNA1FOR DIAG-2       | GTCCGGTGGCATGGATCAAA |
| 5        | GNA1REV DIAG         | GTTCGGCATCTGCGCCA |
| 6        | GNA2FOR DIAG-1       | GCCCTGGCCTACCGGAAACG |
| 7        | GNA2FOR DIAG-2       | TTCGCCGGAGTAAAGCT |
| 8        | GNA2REV DIAG         | GGCACCAATAATGGACCTTAC |
| 9        | GNA3FOR DIAG         | GGCCTTGCCCTACTGCAA |
| 10       | GNA3REV DIAG         | GGATAGGAGTGTTGATGAGTG |
| 11       | GB1FOR DIAG          | GTCCCGTCGGCCAGGTCG |
| 12       | GB1REV DIAG          | TGGTTCACTCGTGGAGAAGGG |
| 13       | HPHREV               | TGCTCTTCTACATCTTCTGTC |
| 14       | HPHFOR               | TGTTGAAGATGACTCCGAGATTG |
| 15       | GB1NAT3’FLANK-FWD    | GTAACGCCAGGTTTTCGGCATCGAGCGGTTCCACAGGGGTTGGGCGG |
| 16       | GB1NAT3’FLANK-REV    | CTACAGTGATCGCCCGCTGCCCTGATCGGTCGAGTGGGCCCAGG |
| 17       | GB1NAT3’FLANK-FWD    | CTCCCTCATAATATCTCCCTGATCGGTCGAGTGGGCCCAGG |
| 18       | GB1NAT3’FLANK-REV    | GCGGATAACAAATCTACAGGGAAACACGCAAGAGGGGATCCTGCCCCCTGAGG |
| 19       | PTRPC5’GNB1          | AGACCCTGAGCTACACGAGAGCGCAGACGATGATGATTGAGG |
| 20       | NAT3’GNB1            | GCCCGCGCCATCATGCAAGAGGCGATCCCGGAGGAGCCAGATCATGAGG |
| 21       | GB1NAT-REV-DIAG      | TCACCTGATGACTCTGGTACATA |
| 22       | NAT 5’ REV DIAG      | CAAAAGTGCTCCTCTCATA |
| 23       | pCCG1 FWD            | CCATCTCAGCCACAAACAG |
| 24       | GNA1ORF REV          | GGAATCTCTCAGAATAGTGG |
| 25       | GNA2ORF REV          | GGAATCTCTCAGAATAGTGG |
| 26       | GNA3ORF REV          | GGAATCTCTCAGAATAGTGG |
| 27       | F-C2                 | CCACTCTTCTACACCGGTCAGCAGTCTCACCTCTAAGG |
| 28       | R-C2-V5G             | GTTAGGGATAGGGTTCTCGCCGCTCCCGCAGGCGGAGGACGATGACACACACAG |
| 29       | P-CPC-2-REV          | TATGCTGATGTTACGGCGGCTGGCAGTTAAGGGGAGGGGAGCACGACACACACAG |

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pRS426 [52], with methods previously described [53]. Primers used to amplify fragments for the construct are listed in Table 2. Primer pairs 15–16 and 17–18 were used to amplify the 1 kb 5’ and 3’ flanks of *gnb*-1, respectively, from genomic DNA. Primers 19 and 20 were used to amplify the nourseothricin resistance marker from plasmid pD-NAT-1 [51]. The three purified PCR products plus pRS426 digested with *Xho*I and *Eco*RI were transformed into yeast strain FY834 [54]. Transformants were selected on FGS plates containing nourseothricin and then checked for the presence of the Δ*gnb*-1::*nat*R mutation using diagnostic PCR with genespecific primers (Primers 21 and 22; Table 2). Positive strains were then purified to homokaryons using serial streaking of macroconidia [47] and checked again using diagnostic PCR (S1 Fig).

Vectors containing predicted GTPase-deficient, constitutively activating mutations *gna*-1*Q204L* (pSVK51), *gna*-2*Q205L* (pSVK52), and *gna*-3*Q208L* (pSVK53) were previously made using site-directed mutagenesis [55]. Electroporation of *N. crassa* with 1–2 μg of pSVK51, pSVK52 or pSVK53 was as previously described [56], using the Δpc2his3A strain as the recipient, with selection on FGS plates without histidine. Genomic DNA was extracted from transformants and subjected to Southern analysis for *gna*-1, *gna*-2 and *gna*-3 as described [55]. Transformants determined to have a single integration event of the transforming DNA at the *his*-3 locus were purified to homokaryons using microconidiation [57] or serial streaking of macroconidia [47] on FGS plates lacking histidine. Genomic DNA was extracted from these strains and analyzed using diagnostic PCR (S1 Fig) to confirm genotypes.

A vector was produced to allow expression of a GFP-tagged version of *cpc-2* in trans to the wild-type copy. The vector backbone (pRS426PVG) [58] was assembled in plasmid pRS426 using yeast recombinational cloning [53]. The fragments included a region 1kb 5’ of the pan-2 ORF, the cgg-1 promoter amplified from pMF272 [59], a multiple cloning sequence, a 5xGlycine linker, a V5-tag, GFP sequence amplified from pMF272 [59], the bar gene, amplified from vector pTJK1 [60] and a 1 kb fragment 3’ of the pan-2 ORF [58]. All fragments were amplified using Phusion High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA). The pan-2 flanking sequences allow targeting to, and deletion of, the pan-2 ORF, resulting in pantothenate auxotrophy. The final expression construct for CPC-2 was produced by insertion of the *cpc-2* ORF (amplified using Primers 27 and 28; Table 2) into vector pRS426PVG (linearized using *Pac*I) using yeast recombinational cloning [53]. Vector pRS426PVG-CPC2 was transformed using electroporation into *N. crassa* strain 51-IV-4 (Table 1) [58]. Transformants were selected on medium containing phosphinothricin and pantothenate [58] and screened for the presence of the integrated DNA at the pan-2 locus using PCR. Positive strains were crossed to wild-type strain 74-OR23-1VA, and ascospores were plated on medium containing phosphinothricin and pantothenate. Progeny were screened for pantothenate auxotrophy by spot-testing and for the presence of the integrated DNA using diagnostic PCR (S1 Fig). Strain CPC-2-GFP-9-10 was selected for further study (Table 1).

A *cpc-2* complemented strain was obtained by crossing the transformants expressing GFP-tagged *cpc-2* described above to Δ*cpc-2* mutant strain Δcpc2#11 (Table 1). Ascosores were plated on FGS plates containing hygromycin and pantothenate to select strains carrying the Δcpc-2 mutation. Progeny were spot-tested on medium containing phosphinothricin and pantothenate, followed by diagnostic PCR (S1 Fig), to determine those that also carried the *cpc-2* GFP trans gene construct at the pan-2 locus. Positive strains were tested for the presence of the CPC-2 GFP fusion protein using western analysis with CPC-2 antiserum as described below. Strain CPC-2-GFP-13.2 was selected for further analysis (Table 1).
Purification of a CPC-2 fusion protein for production of a polyclonal antiserum in rabbits

CPC-2 was expressed as an in-frame, N-terminal Maltose Binding Protein (MBP) fusion protein in *E. coli* and then purified and used as an antigen for antibody generation in rabbits. The cpc-2 ORF was cloned as an *EcoRI*-*PstI* fragment in *E. coli* vector pMAL-c2X (New England Biolabs). The MBP-CPC-2 fusion protein was expressed in *E. coli* strain K12 ER2508 (New England Biolabs) with induction using 300 μM IPTG (isopropyl β-D-1-thiogalactopyranoside; Sigma) and the fusion protein purified using an amylose resin according to the manufacturer’s recommendations. A polyclonal antiserum specific for the MBP-CPC-2 protein was raised in rabbits by Cocalico Biologicals, Inc. (Stevens, PA, USA).

Western analysis to confirm genotypes and check protein levels in mutants

Western analysis was used to check strains for expression of CPC-2 and the G protein subunits GNA-1, GNA-2, GNA-3 and GNB-1. For confirming genotypes, submerged cultures were grown, frozen in liquid nitrogen and then pulverized in 2-ml tubes with metal beads using a TissueLyser (Qiagen Retsch GbmH, Hannover, Germany) as previously described [47]. Subsequently, 500–800 μl of extraction buffer (10mM TrisCl pH 7.5, 0.5 mM EDTA, 0.1% Fungal Protease Inhibitor Cocktail (FPIC), 1 mM PMSF and 1mM DTT) was added to the tube, the solution was mixed and then centrifuged at 5000 x g for 10 min at 4°C. Protein concentration was determined using the Bradford Protein Reagent Concentrate (Bio-Rad, Hercules, CA). Approximately 50 μg of supernatant protein (whole cell extract) was loaded onto a 10% SDS-PAGE gel and then transferred to a nitrocellulose membrane (GE Water and Process Technologies) [61]. For checking G protein levels in the Δcpc-2 mutant, cultures were grown and the protein fraction enriched in plasma membranes was isolated as previously described [62]. For determining CPC-2 protein amount in the G protein mutants, whole cell extracts were isolated as previously described [62]. The protein concentration in the preparations was determined using the Bradford Protein Reagent Concentrate. Aliquots containing equal amounts of protein were subjected to SDS-PAGE, and a western blot was prepared as described above for confirming genotypes of G protein subunit mutants.

Western blot membranes were reacted with the CPC-2 antibody at a dilution of 1:1000 or antiserum raised against GNA-1, GNA-2, GNA-3 or GNB-1 at dilutions of 1:2000 [55, 56, 63, 64]. Blots were then incubated with a goat anti-rabbit antibody horseradish peroxidase conjugate (Bio-Rad; 1:10,000 dilution). Chemiluminescent detection was performed as previously described [61] using the Super Signal West Pico Plus kit (Thermo Fisher, Rockford, IL). Western blots presented in figures are representative of three biological replicates.

Phenotypic analysis

Quantitative assays for aerial hyphae height and growth rates of basal hyphae and qualitative analysis of female fertility were performed as described previously [6, 65]. Twelve biological replicates were obtained for aerial hyphae height and four were used for basal hyphae growth rate calculations. Investigation of hyphal morphology and conidiation in submerged cultures and conidial germination on solid medium were conducted as described previously [55, 66] and the results shown are representative of 2–3 biological replicates. Because the Δcpc-2 gna-5^Q208L strain KAB3210 does not produce appreciable macroconidia, 200 microliters of packed aerial hyphae were used to inoculate submerged cultures for this strain. For quantifying macroconidia, strains were inoculated in 13x100mm glass slant tubes containing 3 ml of VM agar medium and incubated for 4 days in the dark at 30°C and 3 days in light at room
temperature. Macroconidia were collected from tubes by adding 2 ml water, mixing vigorously using a vortex mixer and filtering through Handiwipes into a 15 ml conical tube using a small funnel. This step was repeated twice, once using a wooden stick to dislodge residual macroconidia from the glass tube prior to vortexing and filtering. Macroconidia were pelleted by centrifugation and the water aspirated. Water was added to an appropriate volume and the absorbance read at 600nm using a spectrophotometer. The readings for different strains were all normalized to the same volume (1 ml) to yield a macroconidial concentration expressed as OD600/ml. Eight biological replicates were obtained.

GraphPad Prism 6.0 (GraphPad Software Inc., La Jolla, CA) was used to analyze quantitative traits (hyphal growth rate, aerial hyphae height and conidia abundance). Grubb’s Q test was utilized to detect and eliminate outliers and then the Ordinary One-Way ANOVA test was used for detecting statistical significance. The p-value cutoff was set to 0.05, confidence intervals were 95% and pair-wise comparisons were made. Graphs were created using Microsoft Excel (Microsoft, Redmond, WA).

**CPC-2 localization experiments**

Two approaches were undertaken to determine the intracellular localization of the CPC-2 protein: cell fractionation studies using centrifugation with a wild-type strain and live-cell microscopic imaging of a strain that produces GFP-tagged CPC-2. Cell fractionation of a whole cell extract of strain 74-OR23-IVA (Table 1) was performed as described [55]. Fractions containing whole cell extract, cytosol, and the particulate fraction (membranous organelles and large macromolecular structures) were isolated. The volumes of the cytosol and particulate fractions were adjusted to the same total volume as the original whole cell extract to allow comparison. The protein concentration of the whole cell extract was determined using the Bradford Protein Reagent Concentrate (Bio-Rad). Aliquots containing a volume identical to that containing 50 μg of protein from the whole cell extract were subjected to SDS-PAGE and gels were blotted onto nitrocellulose membranes. Antibody to arginase/AGA (cytosolic marker) [67] was used at a dilution of 1:10,000 and the plasma membrane ATPase/PMA-1 (plasma membrane marker; gift from Kenneth Allen and Clifford Slayman) [68] was used at a dilution of 1:3000. Westerns shown in figures are representative of four biological replicates.

Fluorescence microscopy of the CPC-2-GFP-9-10 strain was conducted essentially as described [66]. The germinating conidia were visualized using differential interference microscopy on an Olympus IX71 inverted microscope (Olympus America) with a 60X oil immersion objective. For visualization of GFP fluorescence, the GFP laser was used for excitation at 400 nm. Images were captured using a QIClickTM digital CCD camera (QImaging Surrey, British Columbia, Canada).

**Results**

*N. crassa* CPC-2 is homologous to predicted RACK1 proteins from other fungi

*N. crassa* CPC-2 is 316 amino acids in length and was previously reported to have 70% percent identity with RACK1 proteins [33]. *N. crassa* CPC-2 and GNB-1 each possess seven WD-40 repeats and share 39% similarity and 24% identity at the protein level. In order to investigate the relationships between CPC-2, GNB-1 and RACK1 and Gβ subunit proteins from other fungi, we subjected orthologous sequences from 18 fungal species to multiple sequence alignment and tree rendering (See Materials and methods for details). Gβ and RACK1 orthologs from the plant *Arabidopsis thaliana* were included as outgroups for the analysis. The fungal
species include representatives from the Ascomycota (nine species), Basidiomycota (four species), Chytridiomycota (two species) and Mucoromycota (three species) [40]. Two of the species from the Mucoromycotina possessed multiple orthologs of both GNB-1 and CPC-2, and all proteins were included in our analysis.

The results for the Gβ group showed that the proteins from *N. crassa* and the other Ascomycete filamentous fungi (*Sordaria macrospora, Fusarium graminearum, Botrytis cinerea, Magnaporthe oryzae* and *Aspergillus nidulans*) cluster together and are more closely related to proteins from Basidiomycetes (*Ustilago maydis, Cryptococcus neoformans, Sporisorium reiliani*um and *Puccinia graminis f. sp. tritici*), Chytridiomycetes (*Batrachochytrium dendrobatidis* and *Spizellomyces punctatus*) and Mucoromycetes (*Phycomyces blakesleeanus* and *Mucor circinelloides f. lusitanicus*) than to the three Ascomycete yeasts (*Saccharomyces cerevisiae, Candida albicans* and *Schizosaccharomyces pombe*) (Fig 1A). These relationships are in keeping with our previous observations that a heterotrimeric Gα subunit from *N. crassa* (GNA-3) is more closely related to proteins from filamentous Ascomycetes and Basidiomycetes than to those from *S. cerevisiae* or *S. pombe* [69]. We also noted that each of the GNB-1 orthologs from *M. circinelloides f. lusitanicus* cluster with 1–2 orthologs from *P. blakesleeanus*, consistent with an ancient duplication event in an ancestor of these two species and later divergence (Fig 1A). Evidence supporting genome duplication in these species has been previously published [70].

In contrast to the Gβ orthologs, the RACK1 proteins distribute into two major clades, with one corresponding to all of the Ascomycetes (including *N. crassa*) and the other containing the Basidiomycetes, Mucoromycetes and Chytridiomycetes (Fig 1B). In the case of the two Mucoromycete species, the RACK1 proteins from each species have the other protein from the same species are their closest neighbor on the tree (Fig 1B). This suggests a more recent gene duplication event for the RACK1 orthologs that occurred after divergence of these two species.

**CPC-2 is a cytoplasmic protein**

We utilized two independent methods to assess subcellular localization of CPC-2. First, differential centrifugation was performed on protein extracts from wild type and the fractions subjected to western analysis using antibodies to marker proteins and CPC-2. Since there was no antibody for CPC-2 available prior to our study, we first expressed and purified an MBP-CPC-2 fusion protein from *E. coli* and used the protein to produce polyclonal antisera in rabbits (see Materials and methods for details). Tests of the serum showed that it recognized a protein of the predicted molecular mass of CPC-2 (~35 kDa) in whole cell extracts from wild type. For the differential centrifugation approach, we generated whole cell extracts, and samples enriched for cytosol and the particulate fraction (membranous organelles and large macromolecular assemblies). Western analysis was performed using antibodies directed against arginase/AGA (cytosolic marker) [67] and the plasma membrane ATPase/PMA-1 (plasma membrane marker) [68], with the results showing good separation of the fractions (Fig 2A). Some contamination of the cytosolic fraction with plasma membranes (but not vice-versa) is evident from the presence of trace amounts of PMA-1 in the cytosol and the absence of the AGA from the particulate fraction. Western analysis using the CPC-2 antibody demonstrated that the great majority of CPC-2 was localized to the cytoplasm, with a small amount in the particulate fraction.

As an alternative method, we implemented fluorescence microscopy to determine the subcellular localization of CPC-2 in a strain expressing a GFP-tagged version of the protein (Fig 2B). The CPC-2-GFP signal was localized in the cytoplasm and excluded from the nucleus (as represented by DAPI staining) in both macroconidia and 6 h germlings (Fig 2B). Thus, both
Fig 2. Subcellular localization of CPC-2. A. Fractionation of CPC-2 during differential centrifugation of cell extracts. Cytosolic and particulate fractions were isolated from a cell extract of wild-type strain 74-OR23-1VA as described in the Materials and methods. Samples corresponding to the same volume of original cell extract were subjected to SDS-PAGE and western analysis using CPC-2, arginase (AGA; cytosol), and plasma membrane ATPase (PMA-1; plasma membrane) antibodies. The results shown are representative of four biological replicates. B. Localization of GFP-tagged CPC-2 protein in vivo. An aliquot containing 8 x 10^6 macroconidia from the CPC-2-GFP-9-10 strain was inoculated on VM agarose plates and incubated at 30°C for 0 h and 6 h. Images for the GFP channel were obtained via fluorescence microscopy and also stained with DAPI to visualize the nucleus (see Materials and methods for details). Images for GFP and DAPI were merged using ImageJ (National Institutes of Health, Bethesda, MD). Differential interference contrast (DIC) images were taken to show overall morphology of macroconidia and hyphae. Scale bar = 10 microns.

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subcellular fractionation and live-cell imaging approaches support a cytoplasmic localization for CPC-2 in *N. crassa*.

**Creation of mutants lacking cpc-2 and G protein subunit genes and analysis of G protein levels in Δcpc-2 strains**

We have previously demonstrated that components of the G protein signaling pathway are crucial for hyphal growth and asexual and sexual development of *N. crassa* [8, 11, 61, 71–73]. In order to explore a possible role for cpc-2 as a heterotrimeric Gβ gene in *N. crassa*, we created strains that could be used for genetic epistasis analysis. We previously employed a similar approach for analysis of genetic relationships between the Gβ *gnb-1* and the three Ga subunit genes [55]. We first purified Δcpc-2 homokaryotic knockout mutants from a transformant created during the Neurospora Genome Project [53, 74] (see Materials and methods). We constructed complemented strains carrying the Δcpc-2 mutation and a *pan-2* targeted, GFP-tagged version of the cpc-2*+* gene (see Materials and methods and Table 1). The complemented strains exhibited significant complementation of several phenotypes, including hyphal growth rate (S2 Fig) and partial complementation of aerial hyphae height (S2 Fig). We used sexual crosses or transformation to generate deletion mutants lacking cpc-2 alone or in combination with mutations in the three Ga genes or the Gβ, *gnb-1*. We also constructed Δcpc-2 strains expressing GTPase-deficient, constitutively activated Ga alleles (*gna-1*Q204L, *gna-2*Q205L or *gna-3*Q208L; see Materials and methods and Table 1).

We have previously shown that, depending on the growth conditions, loss of the Gβ subunit *gnb-1* leads to lower levels of one or all three Ga proteins in *N. crassa* [55, 61, 64]. The exact mechanism underlying this regulation is unknown, but appears to be post-transcriptional, as Ga mRNA levels are normal in Δgnb-1 mutants [61, 64]. Therefore, prior to initiating genetic epistasis experiments with cpc-2, we utilized western blot analysis with protein-specific antisera to check levels of G protein subunits in the Δcpc-2 mutant (Fig 3). The results demonstrate that in contrast to *gnb-1*, loss of cpc-2 does not greatly influence levels of the three Ga proteins or GNB-1 (Fig 3; compare wild type and Δcpc-2 lanes). However, Ga protein levels are still reduced when *gnb-1* is mutated in the Δcpc-2 background (Fig 3). We also consistently noted an increased level of GNA-3 in the Δcpc-2 Δgnb-1 double mutant vs. the Δgnb-1 single mutant, suggesting that loss of cpc-2 partially reverses the effect of the Δgnb-1 mutation. The observation that the Δcpc-2 single mutant has normal levels of G protein subunits greatly streamlines interpretation of genetic epistasis experiments using cpc-2.

We next wanted to determine whether loss of any of the G protein subunits affects CPC-2 protein levels. Because CPC-2 is a cytoplasmic protein (Fig 2), we used protein from whole cell extracts for western analysis using the CPC-2 antiserum (Fig 3). The results demonstrated that CPC-2 protein levels were relatively normal in the G protein single mutants (Fig 3). Thus, similar to the situation with GNB-1 levels in the Δcpc-2 strain, CPC-2 levels are not affected by loss of *gnb-1*; the two predicted Gβ proteins are independent of one another in this regard. Our findings suggest that if CPC-2 does operate as a Gβ subunit, it does not share all functions with GNB-1 in *N. crassa*.

**cpc-2 is epistatic to gna-2 during regulation of basal hyphal growth rate**

*N. crassa* grows by elongation, branching and fusion of hyphae, eventually forming a network structure called the mycelium (rev. in [75]). From this mycelium, aerial hyphae grow upward and spore-forming structures (macroconidiophores) are elaborated from their tips. Formation of cross-walls and constriction of macroconidiophores leads to formation of the mature multinucleated asexual spores, macroconidia. Macroconidia are disseminated in nature by wind
currents, enabling the fungus to colonize new areas. When in the presence of water and suitable nutrients, macroconidia germinate to form a hyphal tube, which then begins the growth program described above [75].

We began our genetic epistasis analysis by investigating the set of mutants for defects in basal hyphae extension rate, using macroconidia to inoculate race tubes (see Materials and methods). The results from genetic epistasis analysis were interpreted as reported previously [55]: If the phenotype of the Δcpc-2, ΔGα double mutant resembles the phenotype of the ΔGα mutant, and if the mutationally activated Gα allele bypasses the phenotype of Δcpc-2, then the Gα gene is epistatic to (implied downstream) to cpc-2. If the opposite is true, then cpc-2 is epistatic to the Gα gene. If contradicting results are seen, this is interpreted as the two genes being partially or completely independent in regulation of the phenotype being assessed.

All of the single gene mutants had a basal hyphal growth rate phenotype (Fig 4, S1 File). In Δcpc-2 mutants, the growth rate was 61% of wild type (Fig 4). The findings from ANOVA of the characterized strains revealed several relationships (S1 File). First, cpc-2 may operate downstream of gna-2. Both mutants are significantly different than wild type, the double mutant grows slower than Δgna-2, but slightly faster than Δcpc-2, and mutational activation of gna-2 (gna-2Q205L allele) does not lead to an increase in growth rate in the Δcpc-2 background. Second, the Δgna-1 and Δcpc-2 knockout mutations are synergistic with regards to reduction in growth rate, and mutational activation of gna-1 does not rescue the Δcpc-2 phenotype; in fact, the growth rate is further reduced in the Δcpc-2 gna-1Q204L strain. The same relationships hold between gna-3 and cpc-2. These results suggest that cpc-2 regulates growth rate using a different pathway than gna-1 or gna-3. Finally, Δcpc-2 and Δgnb-1 are also synergistic, with the double mutant having a significantly slower growth rate than either single mutant (Fig 4).
suggests that these two Gβ-like genes have some independent functions during regulation of hyphal growth in *N. crassa*.

We have previously demonstrated that strains lacking either of the G protein subunit genes *gna-1* and *gna-3*, but not *gna-2* or *gnb-1*, have a defect in germination of macroconidia, an essential step prior to hyphal growth and formation of a colony [66]. Therefore, we explored this phenotype in ∆cpc-2 mutants, using wild type as a control (S3 Fig). Similar to ∆gnb-1 mutants, strains lacking cpc-2 are normal with respect to germination of macroconidia (S3 Fig). Thus, overall colony size of ∆cpc-2 mutants is compromised by slower extension of basal hyphae, and not by a defect in germination of macroconidia.

**cpc-2 is epistatic to gna-2 with regards to aerial hyphae height and ∆cpc-2 mitigates the increased macroconidia production of ∆gnb-1 mutants on solid medium**

We next explored epistatic relationships between cpc-2 and the other genes for two quantitative traits relevant to macroconidiation: aerial hyphae height and macroconidia abundance.

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**Fig 4. Growth rate of basal hyphae.** VM agar race tubes were inoculated with the indicated genotypes, incubated at 25°C and marked at various times as previously described [65]. Linear growth rates were determined with values (expressed as mm/day) taken from four biological replicates. Strains used were 74-OR23-1 VA, ORS-SL6a, 3B10, Agna2-2476, 3c2, 42-8-3, ∆cpc-2#11, C2B1#2-1-1, C2G1#39, C2G2#37, C2G3#1–6, C2G1#44, C2G2#4 and C2G3#1–8 (See Table 1 for genotypes). Error was calculated as the standard error of the mean. ANOVA was performed to identify strains that were significantly different from one another (S1 File).

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Similar to the case for basal hyphae growth rate, all of the single gene deletion mutants had an aerial hyphae height defect (Fig 5A, S1 File). For Δcpc-2, aerial hyphae heights were 69% of wild type (Fig 5A).

ANOVA of the strain set produced results similar to those noted for basal hyphae, above (S1 File). The aerial hyphae height of the Δcpc-2 and Δcpc-2 Δgna-2 double mutants is significantly less than that of the Δgna-2 single mutant and the Δcpc-2 gna-2Q205L strain is similar to the Δcpc-2 single mutant (Fig 5A). This result is consistent with cpc-2 functioning downstream of gna-2 to control aerial hyphae height. In contrast, gna-1 and cpc-2 appear to be independent; the double mutant has shorter aerial hyphae than either single mutant and introduction of gna-1Q204L does not rescue the aerial hyphae defect of Δcpc-2 (Fig 5A). Δgna-3 mutants are shorter than Δcpc-2 and the double mutant is similar to Δgna-3 (Fig 5A). However, the finding that aerial hyphae height is not rescued by the gna-3Q208L allele in the Δcpc-2 background (S1 File) supports independent regulation by these two subunits. Δcpc-2 and Δgnb-1 mutants have similar aerial hyphae height and the double mutant is shorter (Fig 5A; S1 File). As observed for regulation of basal hyphal growth, this finding supports independent signaling by cpc-2 and gnb-1 during control of aerial hyphae height in N. crassa.

Quantitative analysis of macroconidia production in agar slants did not reveal a phenotype for Δcpc-2 mutants (Fig 5B, S1 File). In fact, of the single mutants analyzed, only Δgnb-1 possessed a phenotype (greater conidia production; Fig 5B) and the phenotype of the Δcpc-2 Δgnb-1 double mutant was similar to that of Δcpc-2 (like wild type). This suggests that loss of cpc-2 mitigates the overproduction of conidia observed in the Δgnb-1 mutant, and that cpc-2 is epistatic to gnb-1. For the Gα subunit double mutants, Δcpc-2 Δgna-3 produces fewer conidia than either single mutant and differentiation of macroconidia is nearly halted in the Δcpc-2 gna-3Q208L strain (Fig 5B). These results support independence of cpc-2 and gna-3 during regulation of macroconidiation. A similar situation exists for gna-2, as the Δcpc-2 Δgna-2 double mutant and the Δcpc-2 gna-2Q205L strain produce fewer conidia than either single mutant (Fig 5B). With gna-1, the double mutant is similar to the single mutants, but the Δcpc-2 gna-1Q204L mutant produces less macroconidia, consistent with independence (Fig 5B). The results from analysis of strains carrying the three mutationally activated Gα alleles suggest that all three Gα proteins inhibit macroconidiation when locked in the GTP-bound form.

Δcpc-2 mutants produce macroconidia in submerged cultures

Wild-type N. crassa strains do not differentiate macroconidia while growing in shaken submerged culture unless subjected to heat shock, nitrogen or carbon starvation [76–80]. We have previously demonstrated that loss of the G protein subunits gna-3, gnb-1 and gng-1 leads to macroconidiation in submerged culture under all conditions [61, 64, 69], while Δgna-1 mutants only form macroconidia at high inoculation cell density (≥3x10⁶/ml) in liquid culture [81].

Based on the precedent that the Gβ gene gnb-1 is a negative regulator of macroconidiation in submerged cultures, we analyzed our group of strains for phenotypes at a low inoculation density (1x10⁶/ml). Similar to previous findings, wild type and Δgna-2 mutants do not produce macroconidiophores in submerged culture, while single mutants lacking gna-3, gnb-1 and gng-1 all produce abundant macroconidiophores (Fig 6). Rare macroconidiophores could also be observed in the Δgna-1 strain. We also noted that Δcpc-2 knockout mutants produce macroconidiophores in submerged culture (Fig 6).

Double mutants Δcpc-2 Δgna-1, Δcpc-2 Δgna-2, Δcpc-2 Δgna-3 and Δcpc-2 Δgnb-1 all produce conidia in submerged culture. In all four cases, loss of cpc-2 either leads to submerged
Fig 5. Quantitative phenotypes during asexual development. Strains, error calculations and ANOVA were as in Fig 4. A. Aerial hyphae height. Culture tubes containing liquid VM medium were inoculated with the indicated strains and incubated statically in the dark for three days at room temperature. The distance grown by the aerial hyphae above the medium interface was then measured. Values (mm) were taken from 12 biological replicates. B. Macroconidia production. Macroconidia from the indicated strains were propagated by growth in VM agar culture tubes in the dark at 30°C for four days followed by three days in light at room temperature. Macroconidia were harvested from the cultures and quantitated as described in the Materials and methods. Values represent eight biological replicates.

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conidiation or intensifies the conidiation phenotype of the G protein subunit mutants and the Δgna-3 Δcpc-2 double mutant cultures are mostly conidia (Fig 6). Interestingly, introduction of mutationally activated gna-3 corrects the submerged conidiation phenotype of Δcpc-2, while the corresponding activated alleles of gna-1 or gna-2 do not (Fig 6). This result suggests that

Fig 6. Morphology in submerged culture. Macroconidia isolated from the strains used in Fig 4 were inoculated at a concentration of 1x10^6 macroconidia/ml and cultured in VM liquid medium for 16 h with shaking at 200 rpm in the dark at 30°C. In the case of strain C2G3-1-8 (Δcpc-2 gna-3Q208L), a small volume of aerial hyphae was used to inoculate cultures, as this strain does not produce a significant amount of macroconidia. A sample of each culture was imaged at 40x magnification using DIC (see Materials and methods). Examples of conidiophores and/or free macroconidia are indicated by the white arrows.

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GNA-3 may operate downstream of CPC-2, but also has a CPC-2 independent function in controlling submerged conidiation.

**Constitutive activation of Gα subunits does not restore female fertility to the Δcpc-2 mutant**

*N. crassa* is a heterothallic organism, meaning that a given strain has one of two different mating type genes present at a single genomic locus (idiomorphs; mat A or mat a) [82, 83]. Upon nitrogen limitation, *N. crassa* forms protoperithecia (the female reproductive structures) [75, 84]. In the presence of a male cell (usually conidia) of the opposite mating type, pheromone detection results in chemotropic growth of specialized hyphae called trichogynes from the protoperithecium. The fruiting body, or perithecium, is then formed and contains ascii, each with eight haploid spores (ascospores). Upon maturation, ascospores are ejected from the tips (beaks) of perithecia, in the direction of light. Under laboratory conditions, protoperithecial development can be induced using Synthetic Crossing Medium (SCM), and progeny are obtained from sexual crosses approximately 2–3 weeks post-fertilization [75, 84].

Our previous work showed that the mutationally activated *gna-1 Q204L*, *gna-2 Q205L* and *gna-3 Q208L* alleles were not able to restore fertility to the Δgnb-1 mutant. In fact, introduction of *gna-3 Q208L* resulted in complete inhibition of protoperithecial development, a phenotype that was more severe than that of the Δgnb-1 mutant [55]. Δgnb-1 ΔGα double mutant strains resemble the Δgnb-1 mutant, in that they form protoperithecia, but no perithecia after fertilization [55].

Muller et al. [33] previously reported that *cpc-2* point mutants do not produce protoperithecia and are thus female-sterile. In contrast, our results with the Δcpc-2 knockout mutant indicate some protoperithecia are present, as the cultures produce rare perithecia after fertilization that are mostly submerged in the agar (Fig 7). This phenotype is distinct from that of Δgna-2 and Δgna-3 strains that produce perithecia similar to wild type and from Δgna-1 and Δgnb-1 mutants that do not form perithecia after fertilization (Fig 7).

Inspection of double mutants revealed that Δcpc-2 Δgna-1 strains do not produce visible protoperithecia, perithecia or ascospores (Fig 7), a more severe phenotype than either single mutant. In contrast, Δcpc-2 Δgna-2 mutants resemble Δcpc-2 single mutants. Δcpc-2 Δgna-3 strains exhibit a variable phenotype, with either small, submerged perithecia or no visible perithecia (Fig 7), and no ascospores. Mutational activation of either gna-1 or gna-3 in the Δcpc-2 background leads to no visible protoperithecia, perithecia or ascospores, while activation of gna-2 results in the Δcpc-2 phenotype (Fig 7). These results are consistent with synergy between cpc-2 and gna-1 and gna-3. The phenotype of Δcpc-2 gna-3 Δgnb-1 strains is similar [55], suggesting a common mode of action for gna-3 Q208L and/or interaction between GNA-3 and the two candidate Gβ proteins. In contrast, the different results observed after introduction of gna-1 Q204L into the two mutants hints at a different role for CPC-2 vs. GNB-1 during regulation of female fertility.

As noted previously, the Δgnb-1 strain forms small, aberrant protoperithecia, but no perithecia, upon fertilization [61] (Fig 7). In contrast, similar to Δgnb-1 single mutants, Δcpc-2 Δgnb-1 double mutants do not produce perithecia (Fig 7). This result suggests that gnb-1 is epistatic to cpc-2 during sexual development.

**Discussion**

The *N. crassa cpc-2* gene is not essential and the encoded protein is similar to other RACK1 homologs in fungi. Genetic epistasis between cpc-2 and components of the G protein pathway was performed using double deletion mutants and strains containing Gα activated alleles (see
Fig 7. Phenotypes during the sexual cycle. Strains were ORS-SL6a, 3B10, Δgna2-2476, 3lc2, 42-8-3, Δcpc2#11, C2B1#2-1-1, C2G1#39, C2G2#37, C2G3#1–6, C2G1#44, C2G2#4 and C2G3#1–8 (See Table 1 for genotypes). Macroconidia or hyphae from strains were inoculated onto SCM plates and incubated in constant light at room temperature for 7 days. At that time, half of each plate was inoculated with either macroconidia (males) of opposite mating type or water (control). Males were from wild-type strains 74-OR23-1VA (mat A) or ORS-SL6a (mat a). Incubation was continued under the same conditions for an additional 7 days. The fertilized side of each plate was then photographed using a Leica S8APO stereomicroscope with a DFC280 camera (Leica Microsystems, Buffalo Grove, IL USA). Examples of protoperithecia or submerged, aberrant perithecia are indicated by the black arrowheads, while mature perithecia are shown by the black arrows.

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The results revealed genetic relationships between \textit{cpc-2} and \textit{gna-2} during growth of basal and aerial hyphae, \textit{cpc-2} and \textit{gna-3} during growth in submerged cultures and \textit{cpc-2} and \textit{gnb-1} in regulation of sexual development. In the cases of basal and aerial hyphae growth, the epistatic relationships suggest that CPC-2 operates downstream of the G\textsubscript{\alpha} protein.
implying a tethering function for the Gα in regulation of CPC-2. However, the GNA-3 Gα acts downstream of CPC-2 during submerged culture conidiation, suggesting that the RACK1 protein is holding GNA-3 inactive. CPC-2 appears to operate upstream of the Gβ GNB-1 during sexual development and to act in an antagonistic function during production of macroconidia in agar cultures.

Our investigation of epistasis between the three Gα genes and gnb-1 and cpc-2 revealed some interesting parallels. As mentioned above, the results from the current study suggest that gna-3 is at least partially epistatic to cpc-2 during control of appropriate conidiation in submerged cultures. This is similar to the earlier relationship observed for gnb-1 and gna-3 for this same phenotype, with gna-3 epistatic to gnb-1 [55]. The other two Gα subunits are independent of both cpc-2 (this study) and gnb-1 [55] during the regulation of this trait. This indicates that cpc-2, like gnb-1, is a negative regulator of conidiation in submerged culture, and that only activation of gna-3 offers a genetic bypass mechanism to restore normal hyphal growth. Our previous results from epistasis studies of aerial hyphae height demonstrated that gnb-1 is epistatic to both gna-2 and gna-3 and independent of gna-1 [55]. Together with the current study, the findings are consistent with a model in which the Gβ gene lies downstream of the Gα gene(s) and that gna-1 is independent of both gnb-1 and cpc-2 during aerial hyphae elongation. However, any conclusions based on Δcpc-2 Δgnb-1 double mutants need to be tempered, as loss of gnb-1 leads to decreased levels of the three Gα proteins in all genetic backgrounds tested.

It is intriguing that the Δcpc-2 Δgnb-1 double mutants have higher levels of GNA-3 protein than Δgnb-1 single mutants (but still less than in wild type; Fig 3A). This finding suggests that loss of cpc-2 partially mitigates the effects of the Δgnb-1 mutation. In a canonical model for G protein signaling, GNB-1 would function as a GDI for GNA-3 and loss of GNB-1 might lead to misfolding and/or proteolysis of GNA-3. Mutation of cpc-2 partially counteracts this effect, suggesting that CPC-2 participates in the pathway leading to decreased levels of GNA-3 protein. Furthermore, the finding that Gα single mutants have slower basal hyphae growth rates than wild type and that loss of gnb-1 leads to lower levels of Gα proteins supports a possible tethering function for GNB-1 during hyphal growth. Loss of one Gα protein could free more GNB-1 to bind the other Gα subunits, potentially inhibiting them from serving as positive regulators of basal hyphae growth rate. Along these lines, it has been demonstrated in S. cerevisiae that levels of the Gα protein Gap1p are regulated by ubiquitin-mediated proteolysis, and it has been proposed that this is a mechanism used to modulate levels of the active, free Gβγ dimer during mating [85, 86].

Analysis of the sexual cycle demonstrated that the Δcpc-2 forms rare protoperithecia and perithecia and is therefore female sterile. In contrast, mutants lacking other components of the cross pathway control network (cpc-1 and cpc-3) have normal sexual cycles [87, 88]. This indicates that the sexual cycle defect of Δcpc-2 mutants is not solely due to a defect in the response to amino acid limitation. However, there is a possibility that the two processes may be linked. It has been reported in A. nidulans that amino acid limitation arrests sexual development [28]. Furthermore, loss of the RACK1/cpc-2 homolog cpcB or overexpression of the cpc-1 homolog cpaA also block sexual development, supporting a link between the sexual cycle program and the network that regulates amino acid biosynthesis [28].

Attempts to detect an interaction between CPC-2 and other G protein subunits in N. crassa using the yeast two-hybrid assay were unsuccessful. Presumably due to the large number of binding partners, the difficulty in solubilizing peripheral membrane proteins such as Gα subunits, and protein folding concerns with heterologously expressed proteins, we were also unable to achieve co-immunoprecipitation between CPC-2 and GNB-1 or any of the three Gα proteins using cell extracts or proteins expressed and purified from E. coli. A similar result has
been reported for the RACK1 homolog RAK1 in *U. maydis* [30]. Knowledge of the interactions between RACK1 and G protein subunits is important for full understanding of the biology of G protein signaling. Therefore, experiments such as these and others that investigate the detailed mechanistic wiring that connects CPC-2 to heterotrimeric G protein signaling will be the focus of future work.

**Supporting information**

**S1 Fig. Strain genotyping using PCR.** Strains created in this study were checked for proper integration of the DNA construct at the correct locus via diagnostic Polymerase Chain Reactions (PCRs). Genomic DNA was isolated from the indicated genotypes and used in PCRs with the indicated primers. After electrophoresis, agarose gels were stained using ethidium bromide and imaged. The strains used were 74-OR23-1VA (Wild Type), 3B10 (Δgna-1), Δgna2-2477 (Δgna-2), 31c2 (Δgna-3), 42-8-3 (Δgnb-1), Δcpc2#11 (Δcpc-2, Δmus-52), C2G1*#44 (Δcpc-2 gna-1Q204L), C2G2*#4 (Δcpc-2 gna-2Q205L), C2G3*#1–8 (Δcpc-2 gna-3Q208L), C2G1*#39 (Δcpc-2 Δgna-1), C2G2#37 (Δcpc-2 Δgna-2), C2G3#1–6 (Δcpc-2 Δgna-3), C2B1#2-1-1 (Δcpc-2 Δgnb-1), CPC-2-GFP-9-10 (cpc-2::GFP), and CPC-2-GFP-13.2 (Δcpc-2 cpc-2::GFP). 

- **A. Δgna-1.** Primers #4 and #14 were used to amplify a 1.45 kb band corresponding to the Δgna-1 deletion from the indicated strains.
- **B. Δgna-2.** Primers #7 and #14 were used to amplify a 1.3 kb band corresponding to the Δgna-2 deletion from the indicated strains.
- **C. Δgna-3.** Primers #9 and #13 were used to amplify a 1 kb band corresponding to the Δgna-3 deletion from the indicated strains.
- **D. Δgnb-1.** Primers #21 and #22 were used to amplify a 1.3 kb band corresponding to the Δgnb-1 deletion from the indicated strains.
- **E. ccg-1 promoter-cpc-2 ORF region.** Primers #23 and #29 were used to amplify a 1.0 kb band corresponding to the ccg-1 promoter-cpc-2 ORF region from the indicated strains.
- **F. ccg-1 promoter-gna-1 ORF region.** Primers #23 and #24 were used to amplify a 1.3 kb band corresponding to the ccg-1 promoter-gna-1 ORF region from indicated strains.
- **G. ccg-1 promoter-gna-2 ORF region.** Primers #23 and #25 were used to amplify a 1.4 kb band corresponding to the ccg-1 promoter-gna-2 ORF region from indicated strains.
- **H. ccg-1 promoter-gna-3 ORF region.** Primers #23 and #26 were used to amplify a 1.5 kb band corresponding to the ccg-1 promoter-gna-3 ORF region from indicated strains.
- **I. Δcpc-2.** Primers #1 and #14 were used to amplify a 1.1 kb band corresponding to the Δcpc-2 deletion from the indicated strains.

(TIFF)

**S2 Fig. Analysis of growth rate and asexual development in a complemented strain.** Δcpc-2 complemented strain CPC-2-GFP-13.2 was compared to wild type (WT matA) and Δcpc-2 strain Δcpc2#11 with respect to growth rate of basal hyphae (top; four replicates) and aerial hyphae height (bottom; 12 replicates) on VM medium supplemented with 10 μg/ml pantetheinate. Error is indicated as the standard error of the mean. *** p value <0.001 relative to wild type.

(TIFF)

**S3 Fig. Germination of macroconidia.** Macroconidia were harvested as described in [59]. An aliquot containing 8x10^6 macroconidia was spread on a VM agar plate (100mm plate containing 10 ml agar medium) and spor germination monitored microscopically at 30˚C over the indicated times. DIC (differential interference contrast) micrograph images were obtained using an Olympus IX71 microscope with a QIClick digital CCD camera and analyzed using Metamorph software. Strains used were wild type, Δcpc-2 and Δgnb-1.

(TIFF)
S1 File. ANOVA analysis. Data for basal hyphae growth rate, aerial hyphae height and conidia abundance were analyzed for statistical significance using an Ordinary One-Way ANOVA test with GraphPad Prism 6.0. The p-value cutoff was set to 0.05, confidence intervals were 95% and pair-wise comparisons were made.

(XLSX)

S1 Raw Images. Raw images used for western blots and agarose gels in figures. All images were captured using a CCD camera. A. Western blot used to generate the top panel of Fig 2A. Cytosolic and particulate fractions were isolated from a protein extract of wild-type strain 74-OR23-1VA as described in the Materials and methods. Samples corresponding to the same volume of original cell extract were subjected to SDS-PAGE and western analysis using plasma membrane ATPase (PMA-1; plasma membrane) antibody. Positions of molecular weight markers are indicated along the right side of the blot. The western blot was treated with chemiluminescence solution and exposed for 5 min. The image was flipped horizontally and darkened for the final figure. The results shown are representative of four biological replicates. B. Western blot used to generate the middle panel of Fig 2A. Cytosolic and particulate fractions were isolated from a protein extract of wild-type strain 74-OR23-1VA as described in the Materials and methods. Samples corresponding to the same volume of original cell extract were subjected to SDS-PAGE and western analysis using arginase (AGA; cytosol) antibody. Positions of molecular weight markers are indicated along the right side of the blot. The western blot was treated with chemiluminescence solution and exposed for 1 min. The image was flipped horizontally and darkened for the final figure. The results shown are representative of four biological replicates. C. Western blot used to generate the bottom panel of Fig 2A. Cytosolic and particulate fractions were isolated from a protein extract of wild-type strain 74-OR23-1VA as described in the Materials and methods. Samples corresponding to the same volume of original cell extract were subjected to SDS-PAGE and western analysis using CPC-2 antibody. Positions of molecular weight markers are indicated along the right side of the blot. The western blot was treated with chemiluminescence solution and exposed for 1 min. The image was flipped horizontally and darkened for the final figure. The results shown are representative of four biological replicates. D. Western blot used to generate the top panel of Fig 3. For detection of GNA-1, differential centrifugation was used to isolate the particulate fraction from whole cell protein extracts of the indicated strains. Samples were subjected to SDS-PAGE and western blots prepared. Blots were reacted with antiserum for GNA-1. Positions of molecular weight markers are indicated on the left side of the blot. The western blot was treated with chemiluminescence solution and exposed for 1 min. The image was flipped horizontally for Fig 3. The results shown are representative of three biological replicates. E. Western blot used to generate the second panel of Fig 3. For detection of GNA-2, differential centrifugation was used to isolate the particulate fraction from whole cell protein extracts of the indicated strains. Samples were subjected to SDS-PAGE and western blots prepared. Blots were reacted with antiserum for GNA-2. Positions of molecular weight markers are indicated on the left side of the blot. The western blot was treated with chemiluminescence solution and exposed for 1 min. The image was flipped horizontally for Fig 3. The results shown are representative of three biological replicates. F. Western blot used to generate the third panel of Fig 3. For detection of GNA-3 differential centrifugation was used to isolate the particulate fraction from whole cell protein extracts of the indicated strains. Samples were subjected to SDS-PAGE and western blots prepared. Blots were reacted with antiserum for GNA-3. Positions of molecular weight markers are indicated on the left side of the blot. The western blot was treated with chemiluminescence solution and exposed for 1 min. The results shown are representative of three biological replicates. G. Western blot used to generate the fourth
Panel of Fig 3. For detection of GNB-1, differential centrifugation was used to isolate the particulate fraction from whole cell protein extracts of the indicated strains. Samples were subjected to SDS-PAGE and western blots prepared. Blots were reacted with antiserum for GNB-1. Positions of molecular weight markers are indicated on the left side of the blot. The Western blot was treated with chemiluminescence solution and exposed for 1 min. The results shown are representative of three biological replicates.

H. Western blot used to generate the bottom panel of Fig 3. Protein from whole cell extracts was used to prepare western blots that were then reacted with CPC-2 antibody. Positions of molecular weight markers are indicated along the left side of the blot. The Western blot was treated with chemiluminescence solution and exposed for 1 min. The image was darkened for the final figure. The results shown are representative of three biological replicates.

I. Agarose gel used to generate S1A Fig. Primers #4 and #14 were used to amplify a 1.45 kb band corresponding to the Δgna-1 deletion mutation from the indicated strains. Positions of molecular weight markers are indicated on the left side of the gel. The agarose gel was soaked in ethidium bromide and exposed to UV light for 100 ms.

J. Agarose gel used to generate S1B Fig. Primers #7 and #14 were used to amplify a 1.3 kb band corresponding to the Δgna-2 deletion mutation from the indicated strains. Positions of molecular weight markers are indicated on the left side of the gel. The agarose gel was soaked in ethidium bromide and exposed to UV light for 100 ms.

K. Agarose gel used to generate S1C Fig. Primers #9 and #13 were used to amplify a 1 kb band corresponding to the Δgna-3 deletion mutation from the indicated strains. Positions of molecular weight markers are indicated on the left side of the gel. The agarose gel was soaked in ethidium bromide and exposed to UV light for 100 ms.

L. Agarose gel used to generate S1D Fig. Primers #21 and #22 were used to amplify a 1.3 kb band corresponding to the Δgnb-1 deletion mutation from the indicated strains. Positions of molecular weight markers are indicated on the left side of the gel. The agarose gel was soaked in ethidium bromide and exposed to UV light for 100 ms.

M. Agarose gel used to generate S1E–S1H Fig. Primers #23 and #29 were used to amplify a 1.0 kb band corresponding to the ccg-1 promoter-cpc-2 ORF region from the indicated strains. Primers #23 and #24 were used to amplify a 1.3 kb band corresponding to the ccg-1 promoter-gna-1 ORF region from indicated strains. Primers #23 and #25 were used to amplify a 1.4 kb band corresponding to the ccg-1 promoter-gna-2 ORF region from indicated strains. Primers #23 and #26 were used to amplify a 1.5 kb band corresponding to the ccg-1 promoter-gna-3 ORF region from indicated strains. Positions of molecular weight markers are indicated on the left side of the gel. The agarose gel was soaked in ethidium bromide and exposed to UV light for 50 ms.

N. Agarose gel used to generate S1I Fig. Primers #1 and #14 were used to amplify a 1.1 kb band corresponding to the Δcpc-2 deletion mutation from the indicated strains. Positions of molecular weight markers are indicated on the left side of the gel. The agarose gel was soaked in ethidium bromide and exposed to UV light for 100 ms.

(PDF)

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