



Functional characterization of a Con7-related transcription factor in *Coprinopsis cinerea* indicates evolutionary conservation of morphogenetic roles

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ABSTRACT

Fruiting bodies of mushroom-forming fungi (Agaricomycetes) exhibit the highest degree of multicellular complexity in fungi, yet the molecular underpinnings of their developmental programs remain incompletely understood. Here, we characterize *gcd1*, a gene encoding a transcription factor in the Con7 subfamily of C2H2-type zinc finger proteins. This subfamily has previously been implicated in pathogenic morphogenesis in Ascomycota, but its role in Agaricomycetes has not previously been addressed. In *Coprinopsis cinerea*, CRISPR/Cas9-mediated deletion of *gcd1* resulted in strains with severely impaired fruiting body morphogenesis, with malformed cap, stipe, and gill tissues. *Gcd1* deletion strains lacked universal veil, resembling species with open (gymnocarpous) development. We find that GCD1/Con7 homologs are widely distributed in most Dikarya species and are mostly encoded by a single gene in each species' genome. Transcriptome analyses identified several misregulated genes in the $\Delta gcd1$ mutant, which pinpoint potential mechanisms underlying its developmental defects as well as provided insights into the morphogenesis of mushroom fruiting bodies. These findings establish GCD1 as a key regulator of multicellular development in *C. cinerea* and broaden the known functions of Con7-like transcription factors to include fruiting body morphogenesis in Agaricomycetes. Overall, our results and the morphogenetic role of Con7-like transcription factors of Ascomycota suggest functional conservation over half a billion years of evolution.

1. Introduction

Fruiting bodies of mushroom-forming fungi (Agaricomycetes) represent some of the most complex structures produced by fungi. Their development is governed by a genetically encoded developmental program that involves intricate regulatory mechanisms, most of which are yet to be elucidated. This leaves the basic principles of mushroom development and evolutionary transitions between morphotypes poorly understood. A key challenge in fungal developmental biology is uncovering developmental genes and the regulatory logic that underlies the development of sexual fruiting bodies.

Recent research has made significant progress in documenting the genetics of fruiting body development and uncovered several regulatory genes, mostly transcription factors, that, in various species, regulate

diverse aspects of fruiting. For example, in *Schizophyllum commune* transcription factors have been described that regulate the initiation and number of fruiting body primordia (Ohm et al., 2011; Pelkmans et al., 2017), biomass production, or immune responses within developing mushrooms (Beijen et al., 2025). In *Coprinopsis cinerea* less than a handful of transcription factors have been characterized, which currently cover patchy aspects of fruiting body development, such as light responses (Liu et al., 2022), cap expansion (Muraguchi et al., 2008; Sakamoto et al., 2024), gill formation (Masuda et al., 2016) and spore morphogenesis (Hou et al., 2025). Despite these advances, current knowledge is patchy in any model mushroom species and lacks key details on the differentiation process within mushroom initials.

In a typical mushroom-forming fungus, such as the model species *C. cinerea*, development proceeds from undifferentiated hyphal knots

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(also called aggregates or initials), through several primordium stages in which tissue types - e.g. caps, gills, hymenium and stipe are established in a protected environment, to mature fruiting bodies that sporulate (Kües, 2000; Nagy et al., 2023). However, there is a large diversity in how fruiting bodies develop in various species. For example, early developmental events in some Agaricales species are exposed to the external environment (called gymnocarpic or exocarpic development) while in others basic tissues are established within a primordium that is ensheathed by protective veil tissues (called angiocarpic development (Cléménçon et al., 2004; Cléménçon, 2005). Among model species, the ink cap mushroom *C. cinerea* represents an example of angiocarpy, with both partial and universal veil covering primordia, whereas *S. commune* is a gymnocarpic species with a unique developmental process that evolved via the aggregation of simplified, open cup-like (so called cyphelloid) fruiting bodies. Gymnocarpy was shown to be ancestral in the Agaricales, whereas angiocarpy evolved several times independently and likely conferred adaptive advantage that led to higher speciation rates compared to gymnocarpic species (Varga et al., 2022). While gymno- and angiocarpy represent two very basic developmental types, the underlying genetics is currently unknown.

The defining tissue types in gymno- and angiocarpic fruiting bodies are universal and partial veils, which, in early stages, cover the whole fruiting body and connect the cap margin to the stipe (Cléménçon et al., 2004; Nilsen et al., 2024), respectively. In mature fruiting bodies universal veils are recognized as scales, patches or dots on the cap and/or as bag-like sack at the stipe base (called volva). Partial veil is most often left behind as a ring or annulus on the stipe. The presence of veils and the shape, color and size of their remnants are important diagnostic characteristics in the taxonomy and classification of mushroom-forming fungi. They are also subject to strong evolutionary selection (Nilsen et al., 2024) and may be breeding targets in commercially produced species in which sturdy fruiting bodies with a long shelf-life and delayed cap-opening are preferable.

In this study, we examined the function of a conserved, cap-expressed transcription factor, referred to as *gcd1*, in *C. cinerea* and show that it regulates primordium development and is related to gymno/angiocarpy. Deletion of *gcd1* led to defects of fruiting body differentiation, resulting in malformed cap, stipe and gills and the absence of veil tissues. Phylogenetic analysis revealed that *gcd1* is evolutionarily conserved across fungi, from unicellular yeasts to complex multicellular mushrooms. Transcriptomic analysis of our *gcd1* mutant identified biochemical pathways that might explain the observed morphogenetic abnormalities and uncover new elements of fruiting body development. These findings provide new insights into the molecular mechanisms underlying tissue formation, differentiation and developmental types in mushroom-forming fungi.

2. Materials and methods

2.1. Strains and cultures

The homokaryotic strain of *C. cinerea* A43mutBmut pab1-1# 326 strain was cultured in the yeast extract- and malt extract-glucose (YMG) medium at 37°C for 6–7 days under constant light conditions to generate oidia. For fruiting body induction, we followed a synchronized process as described previously (Muraguchi et al., 2015). Strains were cultured on the YMG agar medium with half the glucose content (0.2 % wt/vol) and incubated under constant darkness at 28°C for 5–6 days until the mycelium reached ~1 cm from the edge of the Petri dishes. The Petri dishes were then transferred to white light under 28°C for 2 h, after which they were placed back to the dark for 24 h, followed by 12-h light/12-h dark period at 28°C.

To determine the mycelial growth rate on agar plates, small agar plugs (d = 5 mm) of the WT, $\Delta gcd1$, and $c\Delta gcd1$ strains were inoculated onto YMG (0.2 % glucose) medium and incubated at 37°C in the dark, with 8 replicates per group. Colony diameters were measured every 24 h

up to 96 h, and the growth rate was calculated as colony growth per hour. Statistical analysis of the differences among these strains was performed using one-way ANOVA.

2.2. Targeted deletion and complementation

The plasmid used for targeted gene deletion includes the pUC19 backbone linearized by primers, upstream and downstream homology arms (1000 bp), and the selection marker para-aminobenzoic acid (PABA) fragment amplified from the pMA412 vector (Schmieder et al., 2019). Primers used for the amplification of each fragment are listed in Supplementary Table 1, the plasmid map for gene deletion is shown in Supplementary Fig. 2. The plasmid used for gene complementation includes the pUC19 backbone linearized by primers listed in Supplementary Table 1, the target gene promoter region (1000 bp upstream of the translation initiation codon (ATG)), the gene coding region and 1000 bp terminator region amplified from the genome of *C. cinerea*. The hygromycin B phosphotransferase gene (*hyg*) was used as a selection marker, amplified by using primers listed in Supplementary Table 1. The promoter and terminator sequences used for the *hyg* gene were amplified from the promoter and terminator sequences of the tubulin-encoding gene in *C. cinerea*. To examine the presence of the GCD1 protein, we tagged it with a 3 × Flag tag. Primers used for gene complementation are listed in Supplementary Table 1, the plasmid map for gene complementation is shown in Supplementary Fig. 2.

The CRISPR/Cas9 system was used for gene deletion in *C. cinerea*. For the transformation, the ribonucleoprotein (RNP) complex consisting of Cas9 protein and single guide RNAs (sgRNA) designed at both ends of the target gene, together with the pUC19 (PABA)-plasmid constructed above were delivered into protoplasts of *C. cinerea*. The protoplast transformation of *C. cinerea* was performed following the protocol described by Dörnte (2012) and Pareek et al. (2022). For the complementation transformation, the pUC19 (*hyg*)-plasmid was delivered into protoplasts of *C. cinerea*. The protoplast transformation of *C. cinerea* was similar to that used in gene deletion, with slight modification. Briefly, after delivering the plasmid to the protoplast, the hygromycin B (Thermo Scientific) was added to the petri dishes after 24 h with the final concentration of 100 µg/mL.

The $\Delta gcd1$ mutants were screened by genomic polymerase chain reaction (PCR) with the primers listed in Supplementary Table 1. The PCR products were amplified by the primer pair *gcd1*_GF/GR shown in Supplementary Fig. 2. The size of the PCR product amplified from WT was 1808 bp; however, samples lacking this band were considered mutants, as the gene fragment might have been deleted or multiple plasmid fragment insertions could have resulted in a band too large to be amplified. *gcd1* complementation transformants were screened with the insertion of the target gene fragments starting from the promoter till the end of the terminator. The PCR primers were designed to amplify 3069 bp genomic fragments of *gcd1* complementation transformants (Supplementary Fig. 2).

2.3. RNA-Seq and data analysis

We sampled the hyphal knot ring along with mycelium from the WT strain and $\Delta gcd1$ strain after 2 h of light induction followed by 24 h in darkness, in biological triplicates. Specifically, the strains were cultured on the YMG agar medium with half the glucose content (0.2 % wt/vol), with each Petri dish covered with cellophane. After 5–6 days, Petri dishes were exposed to light for 2 h and then placed back for another 24 h in darkness. The samples were collected as shown in Fig. 5A. Three biological replicates of each sample type were stored at -80°C until RNA extraction. RNA extraction was performed using the Quick-RNA Mini-prep Kit (Zymo Research, USA), cDNA library and pair-end (150 bp) sequencing were conducted by Novogene Co., Ltd (Cambridge, United Kingdom). The raw read quality was checked by FastQC (Andrews, 2010) (version: 0.12.0) and MultiQC (Ewels et al., 2016) (version: 1.19).

Adapters, low quality bases and short reads were trimmed by bbdutk.sh from BBtools 38.92 (Bushnell et al., 2017) following a parameter of qtrim=r1, trimq= 25, minlength = 40. The trimmed reads were mapped to the reference genome using STAR version 2.6.1a_08-27 (Dobin et al., 2013). The aligned reads were counted for each gene in the genome using the summarizeOverlaps function from the GenomicAlignments v.1.22.1 package (Lawrence et al., 2013) using the “Union” mode. Read mapping statistics are shown in Supplementary Table 2. Sample clustering by Pearson correlation coefficients was performed with the transformed count data by Variance Stabilizing Transformation (VST) in the DESeq2 package (Love et al., 2014). Volcano plots were generated using the EnhancedVolcano package 1.27.0 (Kevin Blighe et al., 2025). Genes with > 5 raw counts in ≥ 2 replicates per sample group were kept for further analysis. Differentially expressed genes (DEGs) were identified by using the R package DESeq2 1.48.1 (Love et al., 2014). Genes with fold change (FC) > 1, BH adjusted p-value < 0.01 (Wald test) were considered as upregulated DEGs, and FC < 1, BH adjusted p < 0.01 (Wald test) were considered as downregulated DEGs.

InterPro (IPR) and GO functional annotations were defined using InterProScan version 5.61–93 (Jones et al., 2014), with a p-value cutoff of 0.05. The GO enrichment analysis was performed by R package topGO 2.60.1 (Alexa and Rahnenfuhrer, 2025). The weight01 algorithm was used with Fisher statistics, and the significance level was set to p-value ≤ 0.05 . Putative CAZymes were identified using dbCAN (Zheng et al., 2023) and further categorized based on their substrate specificity as described in previous publications (Sahu et al., 2023). Based on the substrate specificity information, the groups of plant cell wall degrading CAZymes (PCWDEs) and fungal cell wall degrading CAZymes (FCWDEs) were defined by (Hegedüs et al., 2025) (available at mushroomdb.brc.hu). Ribosomal and mitochondrial ribosomal genes were identified based on InterPro (IPR) annotations. Subcellular localization predictions were performed using DeepLoc 2.0 (Thummuluri et al., 2022).

A custom dataset which contains the gene groups for certain processes was created from previous published data (Supplementary Table 3), including genes related to light response, starvation response, aerial mycelium and attached mycelium and fruiting body initiation and tissue specific gene (Hegedüs et al., 2025; Krizsán et al., 2019). A gene set enrichment analysis of the custom dataset was conducted by R package clusterProfiler with the minimal number of genes annotated for testing (minGSSize) was set to 2 and the FDR-adjusted p-value cut-off was set to 0.01 (Wu et al., 2021).

2.4. Quantitative real-time PCR (qRT-PCR) analysis

We sampled primordia measuring 3–4.5 mm in size from both WT and $\Delta gcd1$ *C. cinerea* strains in two biological replicates. RNA extraction was performed using the Quick-RNA Miniprep Kit (Zymo Research, USA). The first-strand cDNA was synthesized with RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA). The qRT-PCR was performed with PowerUp™ SYBR™ Green Master Mix for qPCR (Applied Biosystems, Weiterstadt, Germany). The amplification program started with an initial UDG activation at 50°C for 2 min, polymerase activation at 95°C for 2 min, followed by 40 cycles of 15 s denaturation at 95°C and 1 min annealing at 60°C. The β -tubulin gene was used as the internal control. Three technical replicates per RNA sample were averaged. Primers used for the qRT-PCR analysis were listed in the Supplementary Table 1.

2.5. The generation of the expression image heatmap

The expression image heatmap was generated using an in-house script applied to an unpublished tissue-specific RNA-seq dataset (Supplementary Table 4 and Supplementary Table 3k). Log2-transformed normalized counts were used for the heatmap, and the color scale was normalized from 0 to 1 to represent expression levels of each gene. Biological triplicates were averaged.

2.6. Sample preparation for western blot analysis

The samples used for nuclei enrichment were obtained from primordia at stage 4 of the WT and $\Delta gcd1$ mutant (expressing the GCD1–3xFlag protein). Caps were collected by dissection, as *gcd1* is highly expressed in the cap. Caps were pulverized in liquid nitrogen using mortar and pestle and further homogenized in a nuclei isolation buffer (NIB, 50 mM PIPES/KOH pH 7.0, 1 M hexylene glycol, 10 mM $MgCl_2$, 0.2 % Triton X-100, 1 mM DTT, 1 mM PMSF, 1x protease inhibitor cocktail (cOmplete, EDTA-free, Merck Millipore) and 25 μ M MG132). The homogenate was centrifuged at 200 x g for 2 min at 4°C. The pellet was re-suspended in NIB, and centrifuged at 200 x g for 2 min at 4°C. This step was repeated once. The sample was then filtered through a 40 μ m cell strainer (Avantor), and the filtrate was centrifuged at 200 x g for 2 min at 4°C. The resulting supernatant (served as the total extract) was further centrifuged at 1200 x g for 10 min at 4°C to separate the cytoplasmic fraction (supernatant) from the enriched nuclear fraction (pellet). Samples were supplemented with Laemmli sample buffer, boiled for 5 min, centrifuged for 5 min at 17,000 x g at room temperature and subjected to SDS-PAGE followed by semi-dry transfer to PVDF (Merck Millipore) and western blotting using the following antibodies. Primary antibodies: anti-FlagM2 (1:10,000, Sigma), anti-Histone H3 (1:5000, Abcam) and anti- α Tubulin (1:10,000, Sigma). Secondary antibodies: Goat anti-mouse IgG-HRP (1:10,000, Dako) and Goat anti-rabbit IgG-HRP (1:10,000, Dako).

2.7. Harvesting, fixation and embedding of the primordia

We excised an agar cube bearing hyphal knots or primordia and transferred it to a Half-strength Karnovsky's Fixative (Karnovsky, 1965) consisting of 1 % paraformaldehyde and 2.5 % glutaraldehyde. Samples were incubated overnight at 4°C with rotational agitation. For embedding, the samples were dehydrated by incubating in an ascending ethanol series with 10 %, 30 %, 50 %, 70 %, 90 % and finally 100 % ethanol, respectively with rotational agitation at room temperature. Following this, the samples were embedded in resin TV7100 (Heraeus Kulzer, Wehrheim, Germany). The block was sliced into 10 μ m thick sections by a rotary microtome (Leica HistoCore Autocut R) equipped with a tungsten carbide knife (16 cm, profile-d). The sections were stained by 0.01 % w/v methylene blue (Sigma-Aldrich) and analyzed using a Leica DM 2500 Microscope with a camera attached.

2.8. Phylogenetic analysis of *gcd1*

For the phylogenetic analysis of *gcd1*, we selected 189 published species (Hou et al., 2025) across Agaricomycotina (168 species), Pucciniomycotina (seven species), Ustilaginomycotina (six species) and Ascomycota (seven species from Pezizomycotina, Taphrinomycotina, and Saccharomycotina). The GCD1 homologs were aligned by mafft (version 7) (Katoh and Standley, 2013). Phylogenetic analysis was carried out in RAxML (version 8.2.12) under the CAT + WAG model with 500 rapid bootstrap replicates (Stamatakis, 2014).

2.9. Copy number evolution of proteins from Con7 subfamily and GH30_3 family

To assess the conservation of GCD1, a protein similarity search was conducted using the GCD1 protein sequence (CopciAB_374546) as a query. The search was performed against a dataset of 655 fungal proteomes (G655 dataset) derived from 655 fungal genomes. These genomes were downloaded from JGI and NCBI before October 22, 2024 (Supplementary Table 5). The recent copy numbers of GCD1 were then visualized in the corresponding species tree. To do this, an MMseqs2 search was run in ultrasensitive mode (-s 7.5) to detect distant homologs. Significant hits were defined using an E-value threshold of $1e-5$ (-e 1e-5), retaining up to 1000,000 target sequences (-max-seqs 1000000).

To confirm that the 741 initial hits belong to the same gene family, a second MMseqs2 search was performed using the 741 proteins as queries against the entire G655 dataset. Considering only the best hit per target species, 732 proteins showed at least 97 % overlap with at least one protein sequence in the hit list of the first MMseqs2 search. After manual curation of ambiguous cases, 730 proteins were ultimately retained as members of the GCD1 gene family.

For species tree inference, we used the 277 marker genes identified by Szánthó et al. (2025) as the starting point. Reciprocal Best Hit (RBH) searches were conducted between the CopciAB genome (query) and the G655 dataset (target) to identify single copy orthologs. Multiple sequence alignments were performed using the MAFFT L-INS-i algorithm and trimmed using TrimAl v1.2 with the strict option. Alignments shorter than 150 amino acids or containing fewer than 500 species were discarded, resulting in 221 single-copy clusters comprising 74,376 aligned sites used for tree reconstruction. Phylogenetic inference was performed using IQ-TREE v1.6.12 under the LG+G substitution model and 1000 ultrafast bootstrap replicates. The following constraint was applied for the tree reconstruction based on former topologies (Merényi et al., 2023) (((((((((Mixos1, Neoir1), Mucci2), (Basm2finSC, Entmu)), Olpbor1), Allma1), Ganpr1), Partr), Rozal1_1), Nter).

The conservation of proteins from GH30_3 family was identified in a published dataset (Merényi et al., 2022) based on the protein ID of *C. cinerea* (CopciAB_381637), and the species tree and gene family evolution analyses were obtained from that source.

3. Results

3.1. Identification of a cap-specific transcription factor gene and generation of deletion mutants

As data accumulates on the developmental transcriptomics of Agaricomycetes species (Krizsán et al., 2019; Nagy et al., 2023), we are able to identify conserved genes with tissue-specific expression patterns. To identify cap-upregulated transcription factor genes, we examined data from Krizsán et al. (2019). The transcription factor 374546 in *C. cinerea* A43mutB43mut pab1-1#326 (*C. cinerea* *AmutBmut1*) stood out as a cap-upregulated gene in *C. cinerea*. It was upregulated in the cap in the young fruiting body stage compared to other tissues in the same developmental stage and other stages (Krizsán et al., 2019) (Supplementary Fig. 1). Orthologous genes in three other Agaricomycetes species (*Laccaria bicolor*, *Armillaria ostoyae*, *Lentinus tigrinus*) were also upregulated in caps, relative to other tissue types (Supplementary Fig. 1) based on published data (Krizsán et al., 2019; Ruytinx et al., 2021; Sipos et al., 2017; Wu et al., 2018).

The gene was deleted in *C. cinerea* by using two CRISPR/Cas9 ribonucleoprotein complexes (Pareek et al., 2022). Two gRNAs were designed at both ends of the target gene (Supplementary Fig. 2B), which allowed the deletion of a 1907 bp gene fragment theoretically (Supplementary Fig. 2). The transformants were screened by colony PCR using a primer pair (gcd1_GF/gcd1_GR) designed targeting both ends of the gene (Supplementary Table 1). Transformants that did not show wild-type (WT) bands (Supplementary Fig. 2C) in the colony PCR were further confirmed by RT-PCR using cDNA as the template and gcd1_RT_1F/gcd1_RT_1R as examining primer (for primers see Supplementary Table 1, for the result see Supplementary Fig. 2E). We obtained two independent mutants, which displayed similar phenotypes, so we selected one for the subsequent analyses. To verify that the resulting phenotypes are indeed the consequence of the deletion of the targeted locus, we complemented the *gcd1* deletion by ectopically introducing a FLAG-tagged version of *gcd1* into Δ *gcd1* mutant that resulted the strains Δ *gcd1* ('c' denotes complementation). Complementation of the deletion mutant was first confirmed by colony PCR (Supplementary Table 1 and Supplementary Fig. 2F). The presence of the GCD1 protein was subsequently verified by western blot analysis of nuclei isolated samples from the FLAG-tagged Δ *gcd1* strain (Supplementary Fig. 3).

3.2. The deletion of *gcd1* led to aberrant fruiting bodies

We analyzed vegetative and sexual phenotypes for the Δ *gcd1* deletion mutants. The morphology of the vegetative mycelium of the Δ *gcd1* strain was similar to that of WT, though its growth rate decreased by 9.09 % (one-way ANOVA, *p*-value < 0.001) (Supplementary Fig. 4). Reintroduction of the WT *gcd1* gene into the deletion strain nearly fully restored vegetative growth rate (0.88 % decrease, one-way ANOVA, *p*-value = 0.356) (Supplementary Fig. 4A and 4B).

We observed much stronger phenotypic differences during fruiting body development. In synchronized hyphal knot induction (Muraguchi et al., 2015), both the Δ *gcd1* and the WT strains were able to form a regular hyphal knot ring after two hours of light induction followed by 24 h of dark incubation. The WT hyphal knots developed into primordia with well-defined cap, gill, stipe and universal veil (Fig. 1A, C), and eventually to mature fruiting bodies (Fig. 1B). In contrast, the Δ *gcd1* strain produced irregular, spherical to donut-shaped primordia with a smooth surface that apparently lacked universal veil (Fig. 1A, C). Most Δ *gcd1* primordia developed a lower, disc-shaped base and a spherical apical region, which is discernible also in cross-sections (Fig. 1A, C). The inner structure of the Δ *gcd1* primordia displayed disturbed differentiation with cap-like and stipe-like structures. The Δ *gcd1* strain also developed slower, its primordia developed from 1 to 5 mm within seven days, whereas the WT strain completed sporulation and deliquescence within this time frame (Fig. 1B). Reintroduction of the flag tagged *gcd1* allele successfully rescued this phenotype (Fig. 1B). We also observed that Δ *gcd1* primordia continued to grow until the agar plate desiccated. This suggests that mechanisms that control size in WT are not fully functional in the Δ *gcd1* strain. This also suggests that the Δ *gcd1* strain does not switch to growth by cell expansion, unlike WT *C. cinerea*, which, after the ~P2 stage mostly grows by expanding its cell volume. Rather, the Δ *gcd1* strain might maintain active growth and cell proliferation (see also RNA-Seq results below).

3.3. The Δ *gcd1* mutant lacks veil tissues

Histological sections of primordia with a size of ~1–5 mm from both WT and Δ *gcd1* mutants revealed problems in primordium development (Fig. 2). The WT strain (Fig. 2A) differentiated several cell types (Fig. 2B Panel 1–5), with enlarged cells in the external nodulus (5) and universal veil (1). Stipe cells (4) were aligned in parallel, while gill cells (3) were arranged in a palisade between the cap and the stipe. The Δ *gcd1* mutant (Fig. 2A) showed a reduction in cellular differentiation (labeled 6–10). We recognize rudiments of cap (7), gill (8), stipe (9) and nodulus (10), however, these are malformed and often topologically misplaced relative to their position in WT primordia. At the cap surface, cortical cells (6) are small and narrow, they resemble cap cells, and there is no evidence of cell expansion as expected for universal veil cells. In older Δ *gcd1* primordia the nodulus enlarges disproportionately, leading to a basal disc-like appearance.

To verify tissue identities at the molecular level, we performed quantitative reverse transcription PCR (qRT-PCR) analysis on 3–4.5 mm primordia, using marker genes selected from a high-resolution tissue-specific RNA-Seq dataset (Supplementary Table 4; Supplementary Fig. 5 for expression images; Supplementary Table 1 for primer sequences). Primordia from both the WT and Δ *gcd1* mutants were dissected into upper cap-like and lower stipe-like regions (Fig. 3). A universal veil marker gene (ID: 470115) showed no detectable expression in either part of the Δ *gcd1* mutant, suggesting the absence of veil in the mutant (Fig. 3). In contrast, a cap marker gene (ID: 422976) exhibited high expression in the cap-like region in WT and reduced to about one-third expression of WT in the Δ *gcd1* mutant (Fig. 3). We observed that the gill marker (ID: 391063) gene has some expression in both the cap- and stipe-like region in both the WT and Δ *gcd1* mutant. Gills occupy a small mass ratio of the fruiting body, this may affect the detection of expression from the bulk-tissue samples collected, nevertheless, the result

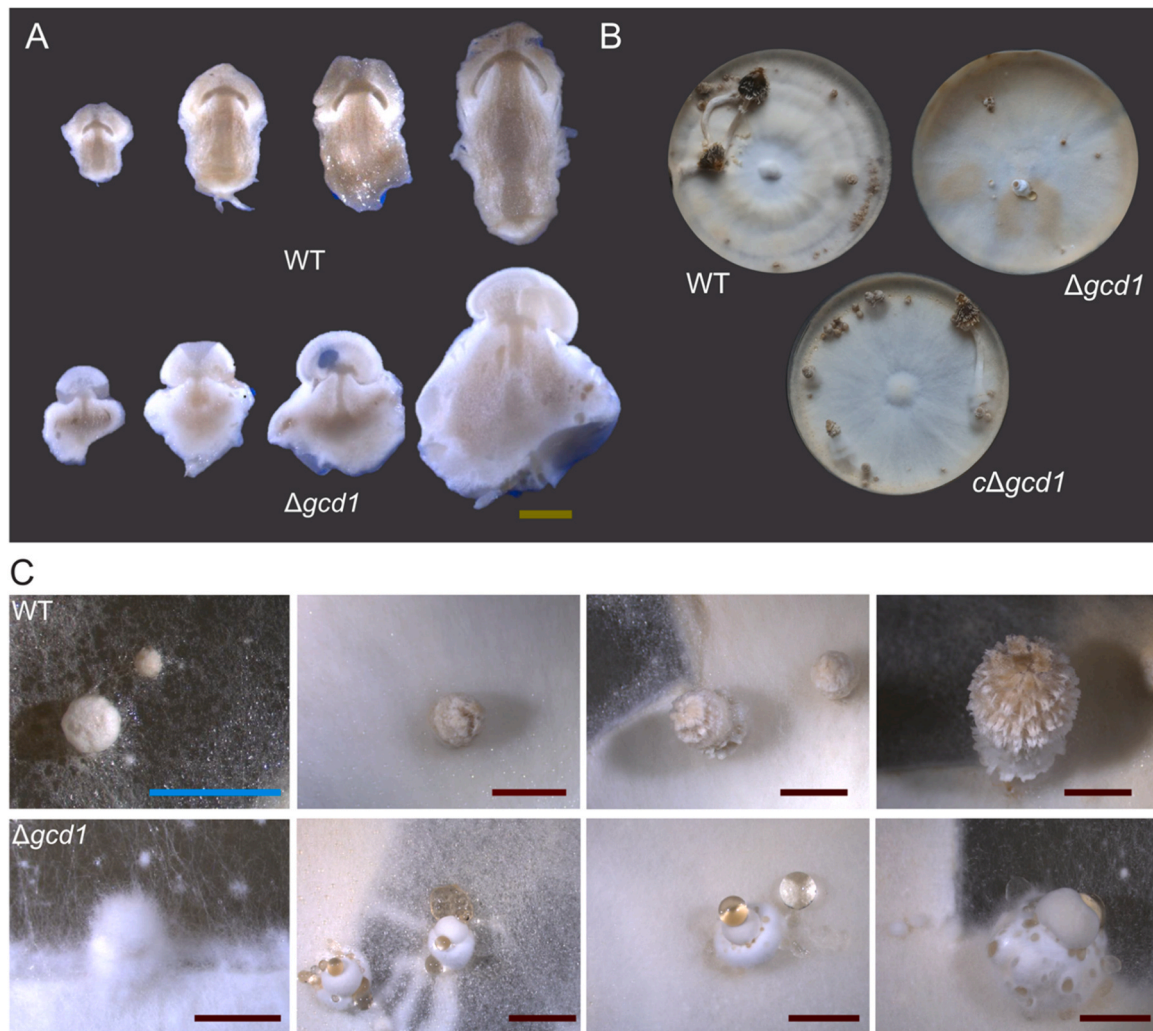


Fig. 1. Fruiting bodies of WT and $\Delta gcd1$ *C. cinerea* strains. A, cross-sections of developing fruiting bodies with similar sizes. Strains were grown on YMG medium with halved glucose content at 28°C; B, colonies 7 days post-light induction, with WT and $c\Delta gcd1$ completing its development whereas $\Delta gcd1$ strain was in a primordium stage; C, successive developmental stages of WT and $\Delta gcd1$ strains. Scale bar = 2 mm.

suggested the presence of gills in the mutant, as also deduced from microscopy observation of sections (Fig. 2B Panel H). The stipe marker gene (ID: 529780) displayed a low and comparable expression level between the WT and $\Delta gcd1$ (Normalized Ct values are listed in Supplementary Table 4) mutants. These results suggested an absence of universal veil cells in the $\Delta gcd1$ mutant and that cap-, gill- and stipe-like cells are likely present in the $\Delta gcd1$ mutant. The absence of veil makes $\Delta gcd1$ mutant primordia gymnocarpic, as opposed to the angiocarpic primordia of the WT. Based on this, we designated *C. cinerea* 374546 as *gcd1*, referring to the gymnocarpic development.

To the best of our knowledge, morphologies similar to what we observed for the $\Delta gcd1$ mutant have not been reported in the literature before and only a few comparable mutants are available. The *ich1* mutant in *C. cinerea* (Muraguchi and Kamada, 1998), also exhibited developmental problems and eventually an arrest; it produced barrel-shaped fruiting bodies but failed to develop a cap. Földi et al. (2024) reported the *snb1* mutant in *C. cinerea*, which formed spherical, rudimentary fruiting bodies that failed to differentiate caps, stipes, and gills. Both the *snb1* and *ich1* phenotypes are, however, different from that of the $\Delta gcd1$ mutant, suggesting that these genes may regulate different aspects of fruiting body development. The *gcd1* mutant seems to develop more tissue types than either previously reported mutant, though in aberrant or rudimentary form, which perhaps preclude continued development towards sporulation.

3.4. GCD1 is a conserved member of the Con7 subfamily of C2H2 zinc finger transcription factors

The *gcd1* gene encodes a 670 amino acid polypeptide which contains one C2H2-type zinc finger domain (IPR013087). Based on its domain content it belongs to a Con7 subfamily (IPR039327) which was first identified in *Pyricularia oryzae* (Odenbach et al., 2007) (as *Magnaporthe grisea*). We analyzed the conservation and gene copy number distribution of *gcd1* by using two datasets, one comprising 655 published genomes (G655) from MycoCosm (Grigoriev et al., 2014) and NCBI (Fig. 4), (Dikarya and non-Dikarya fungi, with Nucleariida and Holozoa as outgroups) and a dataset of 189 species with higher resolution in the Agaricomycetes (Hou et al., 2025).

Our analysis indicates that the Con7 subfamily of transcription factors (i.e. GCD1 homologs) are mostly encoded by a single gene in species that possess it. This was true both in the 655-species (Fig. 4.) and the 189-species datasets (Supplementary Fig. 6). We found the largest variance in copy numbers in the Mucoromycota, where certain species have > 10 copies of the encoding gene.

We found that Con7/GCD1 homologs are prevalent in Agaricomycotina (92 %), Pezizomycotina (91 %), Ustilaginomycotina (79 %) and Pucciniomycotina (80 %). In contrast, only 53 % of the examined Saccharomycotina species and 33 % of the examined Taphrinomycotina species possess this subfamily. Notably, the fruiting-body-

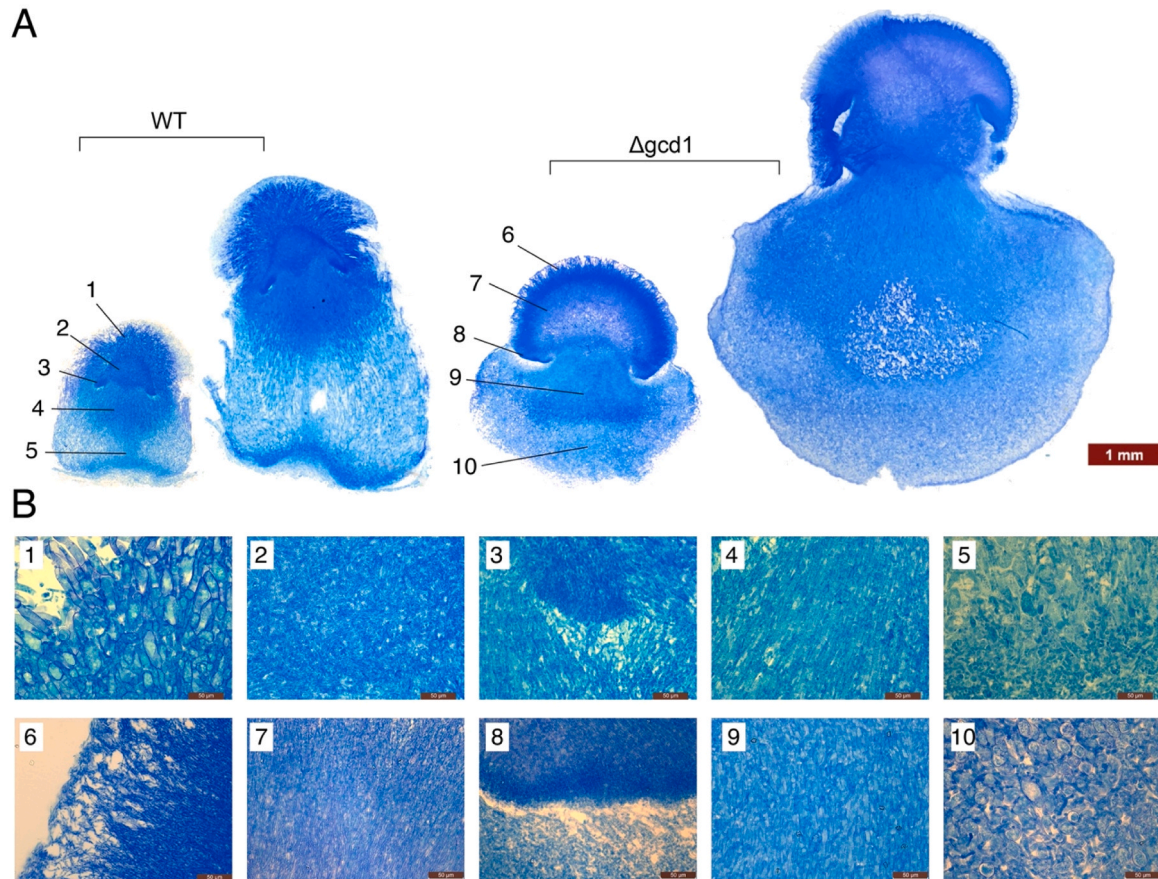


Fig. 2. Histology of WT and *gcd1* mutant strains. A, histological sections of primordium stages from WT and $\Delta gcd1$ stained with methylene blue. B, distinct tissue regions from WT (panel 1–5) and $\Delta gcd1$ (panel 6–10). Tissues are labeled as follows: universal veil (1), cap (2), gills (3), stipe (4), nodulus (5); The labeling in $\Delta gcd1$ (panels 6–10) corresponds to the respective regions in WT. Scale bar = 50 μ m.

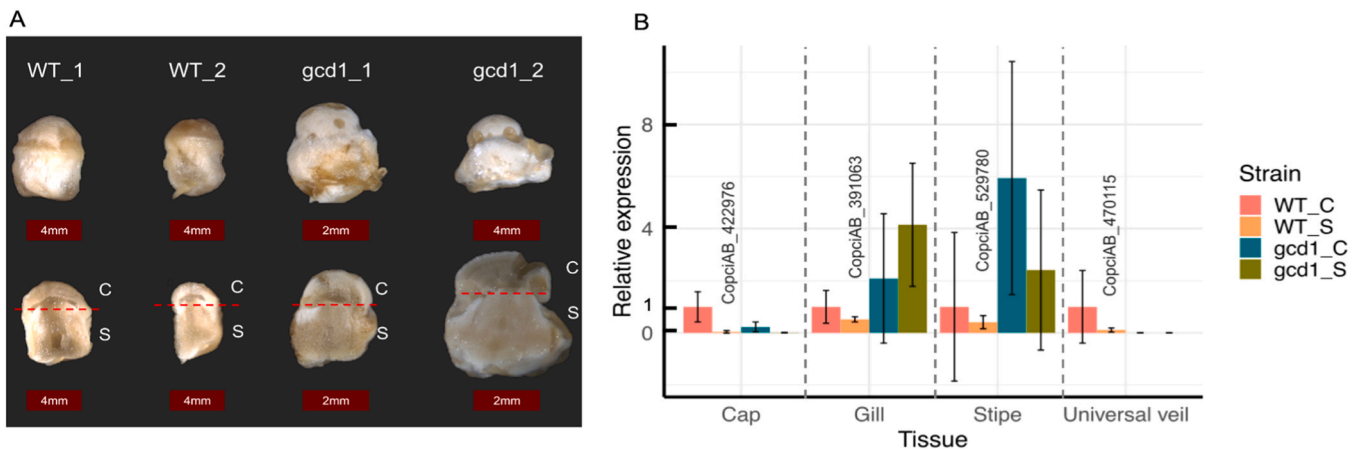


Fig. 3. Quantitative RT-PCR analysis of tissue marker genes by using primordia from the WT and $\Delta gcd1$ mutant. A, primordia used for the qRT-PCR analysis. Primordia were dissected along the lines indicated by red dashed lines into two parts: the upper part (using the letter C to represent), including cap-, gill-, and universal veil-like structures, and the lower part (using the letter S to represent), including stipe-like structures. B, relative expression levels of tissue marker genes in WT and $\Delta gcd1$ mutant strains, determined by qRT-PCR. The beta-tubulin gene has been used as the reference gene. Expression is normalized to the WT cap sample. Three technical replicates per RNA sample and two biological replicates were averaged, and the standard deviation is shown on bars.

forming *Neolecta irregularis* (within Taphrinomycotina), despite being nested among predominantly yeast-like taxa, possesses a GCD1 homolog. GCD1 homologs were rarely present in the examined Zoopagomycota species (17 %) and were not detected in the Holozoa clade and the Rozellida-Microsporidia clade, whereas nearly all Mucoromycota (88 %) have GCD1 homologs. The C2H2 TFs have undergone significant

expansion during fungal evolution (Merényi et al., 2023). Con7/GCD1, as a member of this family, likely arose during this diversification. Its distribution, in fungi and absence in non-fungal lineages, indicates that Con7/GCD1 homologs form a fungal-specific subfamily of C2H2, reflecting fungal-specific evolutionary innovation in transcriptional regulation. To assess its conservation within Agaricomycotina, we

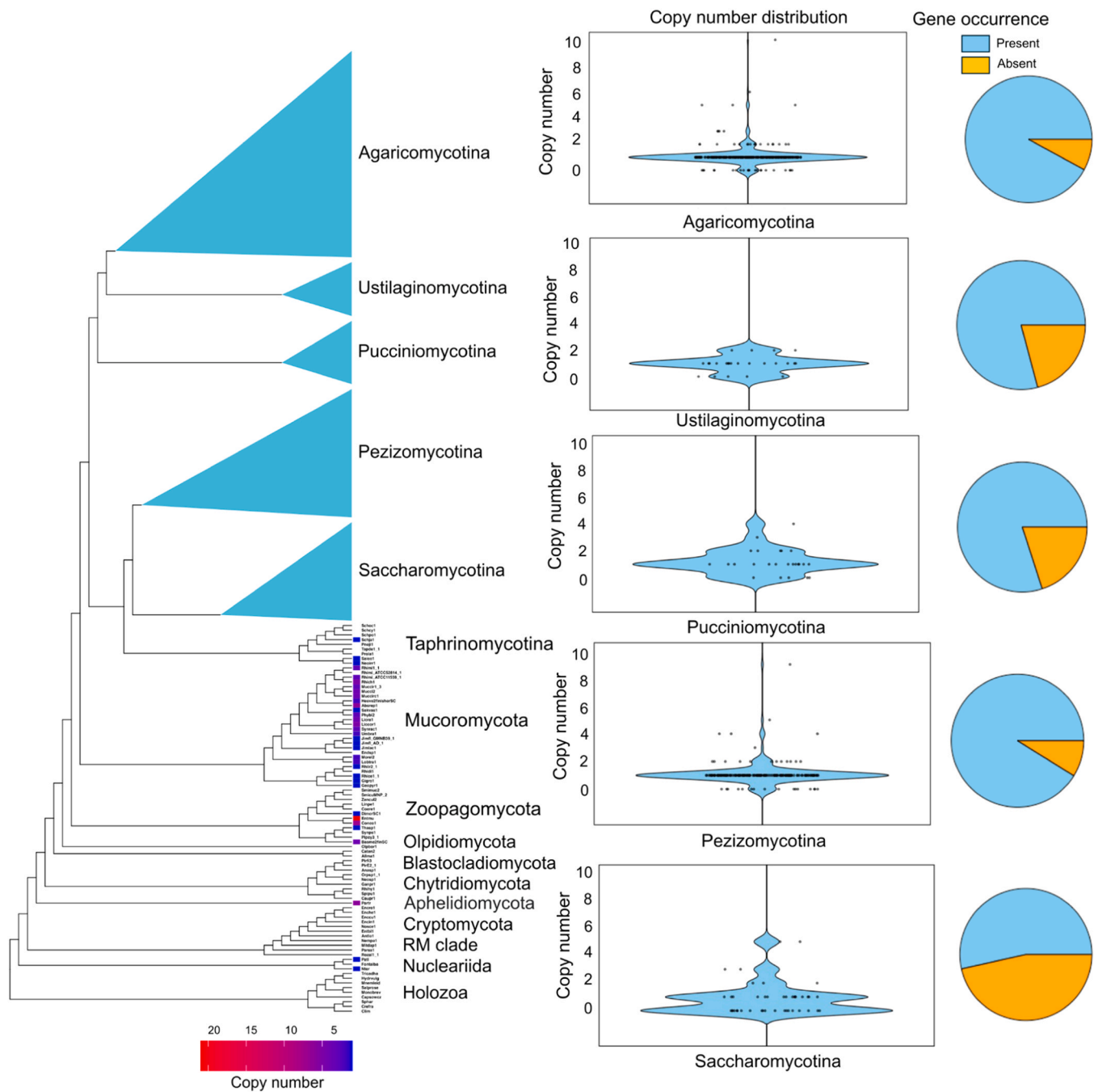


Fig. 4. Phylogenetic distribution of *gcd1* and its copy numbers in G655 dataset. For Taphrinomycotina species and each non-Dikarya species, the copy number of the *gcd1* gene is shown next to the corresponding leaves. Violin plots display the distribution of *gcd1* gene copy numbers within each major clade illustrating variation and central tendency. Pie charts summarize the proportion of species within each clade that possess the *gcd1* gene.

performed a phylogenetic analysis by using GCD1 homologs identified in 189 published genomes (Hou et al., 2025). The gene tree revealed that GCD1 is present in a single copy in most species (Supplementary Fig. 6), in agreement with the comparative genome dataset above. The gene tree approximately resolved main clades in the Agaricomycotina, however, it included several unstable nodes and generally low bootstrap support values. This is due to the high variability of the amino acid sequences in the multiple alignment, which could not be reliably resolved despite multiple alignment strategies tested. Nevertheless, our analysis indicates that the GCD1 is broadly found across the fungal kingdom, is very likely fungal-specific and is encoded by a single copy gene in most species.

3.5. Transcriptomic analysis of the $\Delta gcd1$ and WT strains

The above results indicate that the Con7 subfamily occurs much more broadly (across the Dikarya) than gymno- and angiocarpic fruiting bodies do, suggesting that it regulates more basic functions than fruiting body type. It follows that gymnocarp in the mutant may then be an indirect consequence of the deletion of *gcd1*. To understand how the deletion causes the observed phenotypic differences, we performed transcriptomic analysis by using the hyphal knot stage. We opted for this stage because in the secondary hyphal knot there is minimal morphological divergence between WT and $\Delta gcd1$ strains, which allows us to detect direct effects of *gcd1* deletion better than samples which differ morphologically. Based on previous transcriptomic data (Krizsán et al.,

2019), *gcd1* has a very low expression level in the vegetative mycelium but is highly upregulated in secondary hyphal knots (Supplementary Fig. 1A) which suggests its regulatory impact can be studied at these early stages.

We performed RNA-Seq on the hyphal knot ring from WT and $\Delta gcd1$ strains after 2 h of light induction followed by 24 h in darkness, in triplicates (Fig. 5A). At this stage both WT and $\Delta gcd1$ have hyphal knots with some internal differentiation (Kües, 2000), yet there is no major structural difference between the two sample types that would introduce unnecessarily high numbers of unspecific gene expression differences into the analyses. Pearson correlation coefficients showed good consistency for both WT and $\Delta gcd1$ biological replicates (Fig. 5B). The transcriptomic analysis revealed 935 differentially expressed genes (DEGs) (Supplementary Table 6a and 6b) with a fold change greater than 1 and a BH-adjusted p-value < 0.01 (Wald test). We applied a low fold-change threshold to identify DEGs due to the presence of vegetative mycelium in the samples, which may reduce the relative abundance of transcripts associated with hyphal knots. Of the 935 DEGs identified, 350 were upregulated, while 585 were downregulated (Fig. 5C, Supplementary Table 6a and 6b).

DEGs included few experimentally characterized genes, which drove us to functionally characterize them using computational tools. Exceptions include the cospin *pic1* (Sabotić et al., 2012) (367957) and *keps* (Vogt et al., 2022) (426342 and 503649) among downregulated genes, and *mep3* fungalsin (Lilly et al., 2008) (26022), *cfs1* (Sakamoto et al., 2018) (447925) and the hydrophobin *CoH2* (Ásgeirsdóttir et al., 1997) (468111) among upregulated genes. To computationally characterize DEGs, we used Gene Ontology terms and tested the overlap of $\Delta gcd1$ DEGs with five published gene lists (Hegedüs et al., 2025; Krizsán et al., 2019) describing fruiting body initiation, mycelium type, starvation and light response in *C. cinerea* (Supplementary Table 3). Hereafter we discuss results of these analyses, with a focus on downregulated genes, because we expect these to comprise morphogenesis-related genes directly or indirectly affected by GCD1. We also focus on upregulated genes, which give some clues on potential mechanisms explaining the growth of $\Delta gcd1$ primordia.

Results of Gene Ontology (GO) enrichment analyses are summarized in Fig. 6 and Supplementary Table 7. Overall, 32.14 % and 60.57 % of down- and upregulated DEGs are associated with any GO term, respectively, compared to 47.17 % of all protein coding genes in the genome. This represents a significant depletion of GO terms in downregulated DEGs (Fisher's exact test p-value = 5.67×10^{-14}) (Supplementary Table 7a) and suggests that the functions of downregulated DEGs in the $\Delta gcd1$ mutant are particularly poorly annotated functionally compared to the genome-wide average in *C. cinerea*. In contrast, a higher ratio of upregulated DEGs had GO annotations (Ratio: 60.57 %; Fisher's exact test p-value = 3.88×10^{-7}) than all genes in the genome (Supplementary Table 7a), suggesting that upregulated DEGs include better annotated biological functions, and perhaps more conserved genes.

3.5.1. Downregulated genes

Among the downregulated genes, a total of 19 GO terms were significantly enriched (Fisher's exact test, p-value ≤ 0.05), with 92 genes annotated to these enriched GO terms. Of these we here highlight those related to the fungal cell wall (FCW) (carbohydrate metabolic process, polysaccharide catabolic process, glucosylceramidase activity, fungal-type cell wall and structural constituent of cell wall) and signaling pathways (protein phosphorylation, protein kinase binding) (Supplementary Table 7b).

In association with the cell wall we identified two putative chitin deacetylase genes in the CE4 family (440521, $FC_{wt/\Delta gcd1}$: 4.05; 518489, $FC_{wt/\Delta gcd1}$: 2.53), a GH18 chitinase (488885, $FC_{wt/\Delta gcd1}$: 2.95), four glucanase-encoding genes (366990, $FC_{wt/\Delta gcd1}$: 3.01; 376074, $FC_{wt/\Delta gcd1}$: 2.40; 459642, $FC_{wt/\Delta gcd1}$: 1.80; 524741, $FC_{wt/\Delta gcd1}$: 1.83) belonging to GH16 and one belonging to GH12 (361787, $FC_{wt/\Delta gcd1}$: 2.65), which are likely involved in glucan remodeling. In the context of the complete developmental process of *C. cinerea*, one of the genes (459642) was almost exclusively expressed in the stipe of the mature fruiting body (Krizsán et al., 2019) (Supplementary Table 6b) indicating its potential role in stipe development. Another glucan-related gene (361787) had the highest expression level in caps and gills of the mature

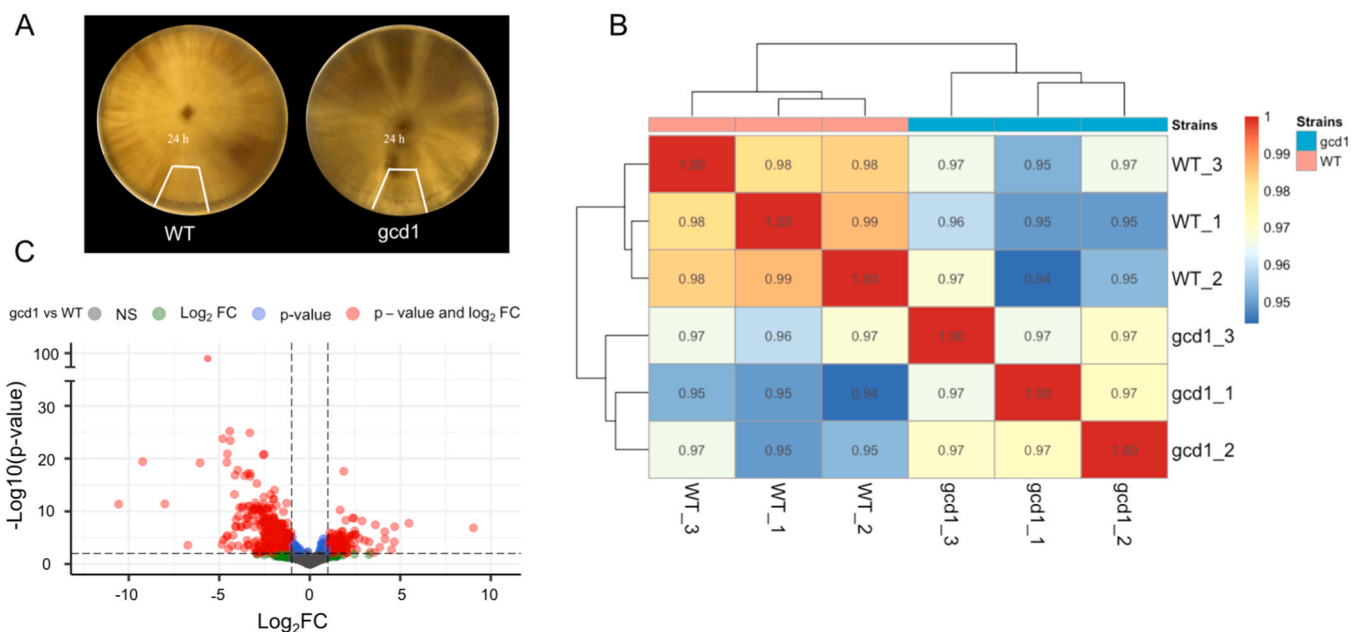
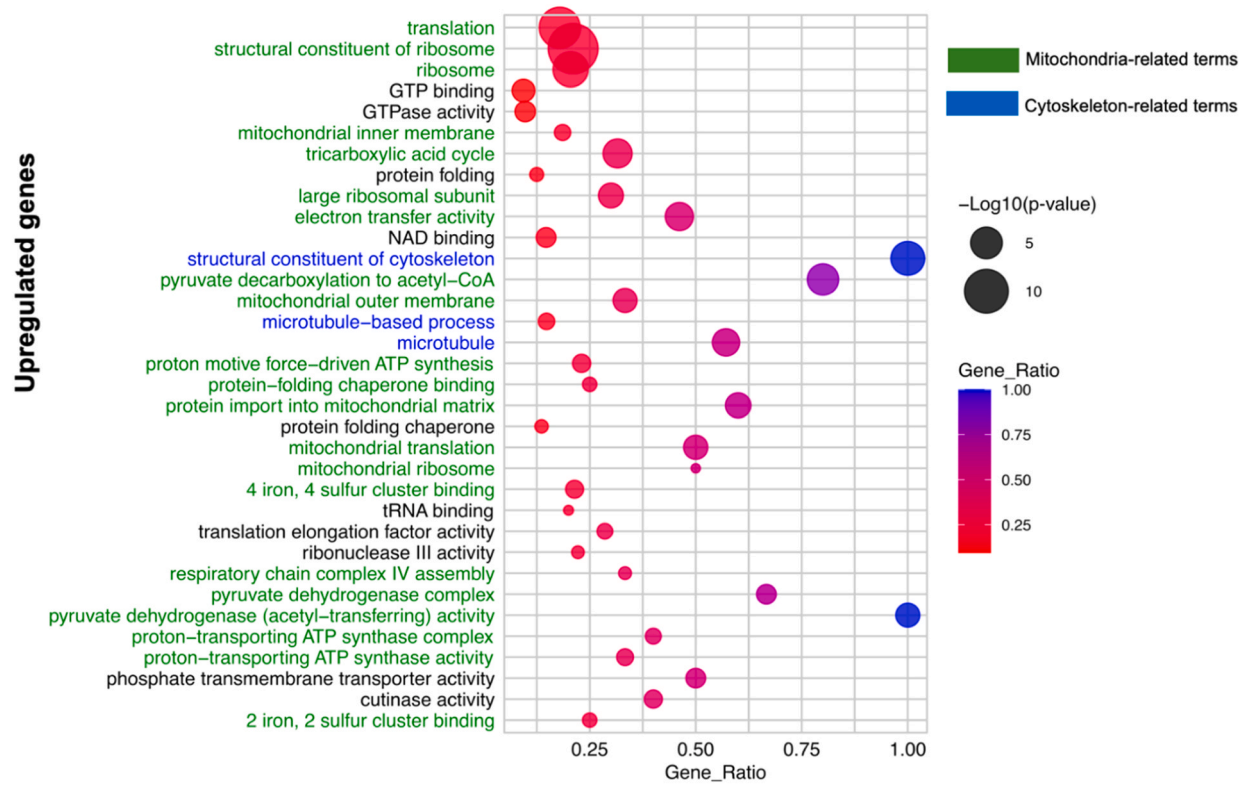


Fig. 5. RNA-Seq analysis of the hyphal knot stage of the $\Delta gcd1$ strain versus WT. A, sampling scheme for RNA-Seq. The sampled region is delineated by a white line; B, heatmap of sample correlations. The numbers in the cell are the Pearson correlations coefficients across samples; C, Volcano plot of the differentially expressed genes. The gray and green color represents non-significant differentially expressed genes (DEGs) with an FDR adjusted p-value ≥ 0.01 (Wald test). The blue color indicates genes with an FDR-adjusted p-value < 0.01 (Wald test) and a log2 fold change (log2FC) between 0 and 1. Red denotes significant DEGs with an FDR-adjusted p-value < 0.01 (Wald test) and a log2FC greater than 1.

A



B

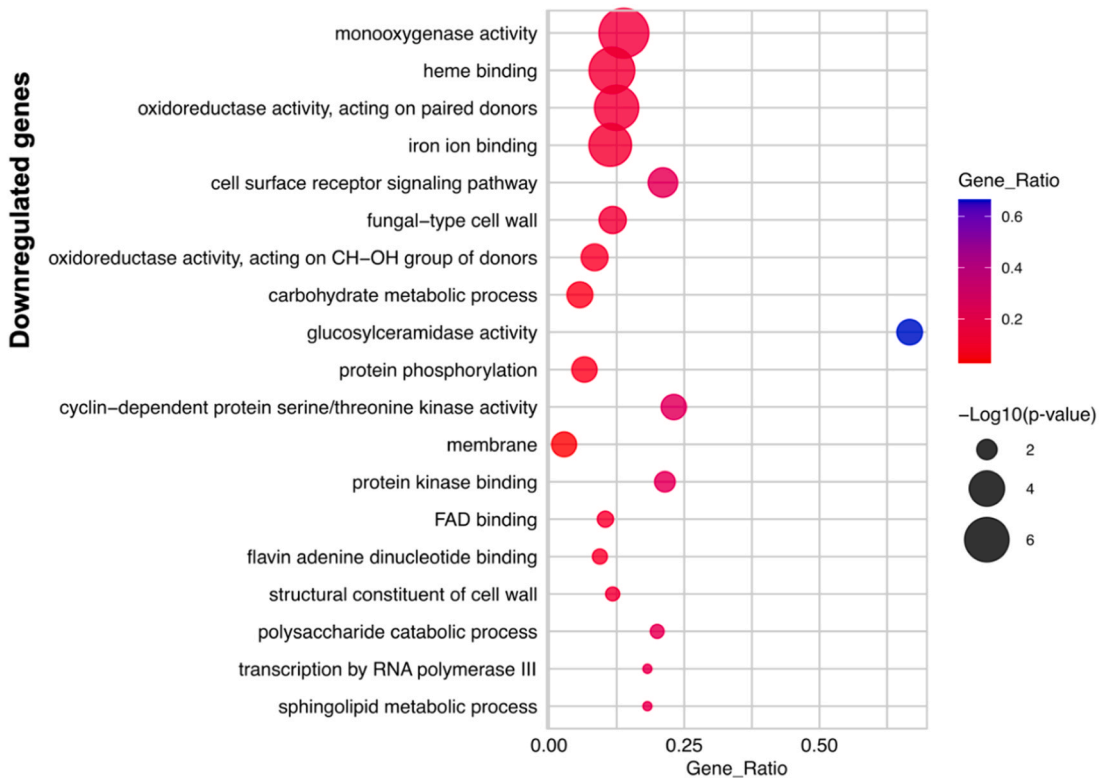


Fig. 6. GO enrichment analysis of the Upregulated DEGs (A) and Downregulated DEGs (B).

fruiting body in *C. cinerea* (Krizsán et al., 2019) (Supplementary Table 6b). Even though we detected these downregulations in the hyphal knot stage, we believe that some of this can explain the morphological defects that we see in the later stages.

We also identified two downregulated genes encoding putatively

glucan-related GH30_3 family proteins (372384, $FC_{wt/\Delta gcd1}$: 2.43; 381637, $FC_{wt/\Delta gcd1}$: 1.69). One of these (381637) has been functionally characterized in *C. cinerea* (as GH30A) and shown to have endo-1,6-glucanase activity (Liu et al., 2020). Its high expression level in mature caps (Liu et al., 2020) (see also Supplementary Table 6b)

suggested a role in autolysis. In *Lentinula edodes*, its ortholog, LePus30A exhibited endo- β -1,6-glucanase activity against fruiting body cell walls and was suggested to be responsible for senescence (Konno and Sakamoto, 2011). The GH30A gene of *C. cinerea* has also been proposed to play a role in cell wall remodeling by replacing β -1,6-glucan to β -1,3-glucan crosslinking in the stipe base, which may reduce wall extensibility, leading to the cessation of growth (Liu et al., 2020). Our results on the $\Delta gcd1$ mutant indicate potential links between the GH30_3 family and early developmental events in *C. cinerea*.

Given the apparent importance of GH30_3 in fruiting body development, we conducted a phylogenetic analysis of GH30_3 protein sequences and inferred their ancestral copy numbers across using 109 species (G109) including Basidiomycota (104 species) and Ascomycota (5 species) using a previously published dataset (Merényi et al., 2022). The examined Ascomycota had very few GH30_3 genes (mean: 0.2, 5 species). In the Basidiomycota, GH30_3 genes occur patchily in non-Agaricomycetes clades (mean: 0.9, 15 species), whereas nearly all examined Agaricomycetes had GH30_3 genes (mean: 2.1, 89 species) (Fig. 7). Ancestral copy number inferences revealed several duplications in Agaricomycetes (see arrows in Fig. 7). Consistent with this, early-diverging orders (Cantharellales to Russulales) have on average 0.9 GH30_3 genes, while Polyporales, Boletales and Agaricales possess 2.1, 2.1 and 2.2, respectively. At the scale of the Agaricomycetes these observations indicate higher GH30_3 copy numbers in orders that possess complex fruiting bodies, although at finer scales the correlation with complexity breaks down (e.g. only two genes in *C. cinerea*, but nine in *Fibulorhizoctonia psychrophila*). Nevertheless, the GH30_3 family seems to be expanded in Agaricomycetes. In addition, one of the genes from GH30_3 family (381637) has been reported to be developmentally regulated among 11 examined species with complex multicellular fruiting bodies (Nagy et al., 2023). Taken together, we speculate the

expansion of genes from GH30_3 may be linked to the evolution of fruiting bodies.

Another group of four downregulated genes linked to the FCW encode hydrophobins (361161, $FC_{wt/\Delta gcd1}$: 3.85; 413238, $FC_{wt/\Delta gcd1}$: 5.61; 419467, $FC_{wt/\Delta gcd1}$: 1.50; 427537, $FC_{wt/\Delta gcd1}$: 11.05). Hydrophobins have been extensively studied in fruiting body development in mushroom-forming fungi (Kim et al., 2016; Ohm et al., 2010; Sammer et al., 2016; Tao et al., 2019), as well as in pathogenicity in plant-pathogenic fungi (Ruiz-Roldán et al., 2015). In plant-pathogenic fungi, deletion of *gcd1* also led to a downregulation of hydrophobin genes (Ruiz-Roldán et al., 2015).

The next significant functional enrichment was associated with signaling pathways (protein phosphorylation (GO:0006468) and protein kinase binding (GO:0019901)) (Fig. 6B). In these categories we found 20 downregulated kinases, of which 13 belong to the fungal specific FunK1 kinases and two to Tyrosine kinase-like (TKL) kinases (Supplementary Table 8). Both FunK1 and TKL kinases have expanded significantly in Agaricomycetes (Krizsán et al., 2019). Eleven out of 20 downregulated kinase-encoding genes were developmentally regulated in Agaricomycetes fruiting bodies (Krizsán et al., 2019), indicating a dysregulation of regulatory signaling pathways may be associated with defective fruiting body development in our mutant.

Gene set enrichment analyses indicated that downregulated DEGs showed significant overlap with genes related to fruiting body initiation, cap-upregulated genes, aerial-mycelium specific genes as well as gene sets defined as late light response and core starvation (Hegedüs et al., 2025; Krizsán et al., 2019) (Fig. 8B). Of these, a high overlap with cap-upregulated genes (gene number/DEGs: 124/585, FDR adjusted p-value = 1.95×10^{-13} , Hypergeometric test; Supplementary Table 3) is of particular interest, given the malformation of caps and the lack of veil in the mutant. We identified two downregulated genes related to

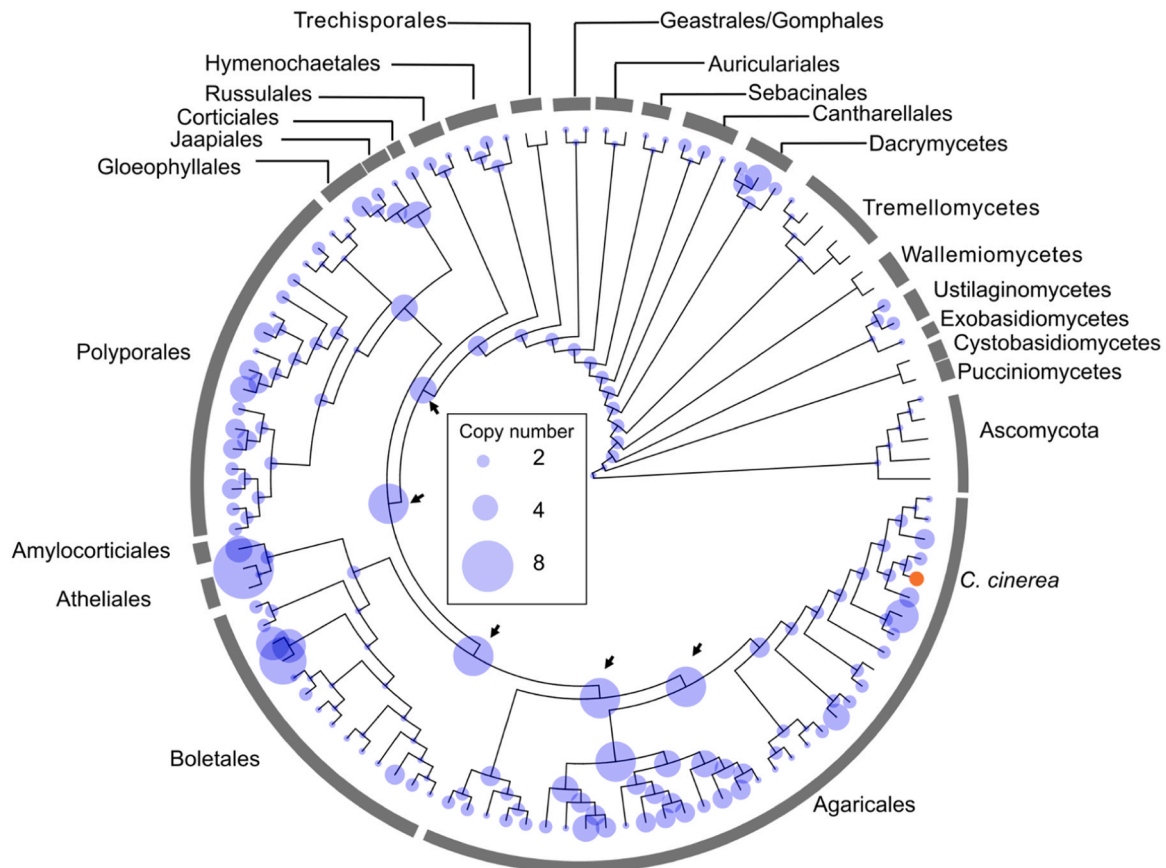


Fig. 7. Species tree of G109 dataset showing extant and inferred ancestral copy numbers of the GH30_3 family in 109 species. *C. cinerea* is highlighted with orange, which has two paralogs (381637 and 372384). The phylogenetic tree and gene family evolution analysis was obtained from a published dataset (Merényi et al., 2022).

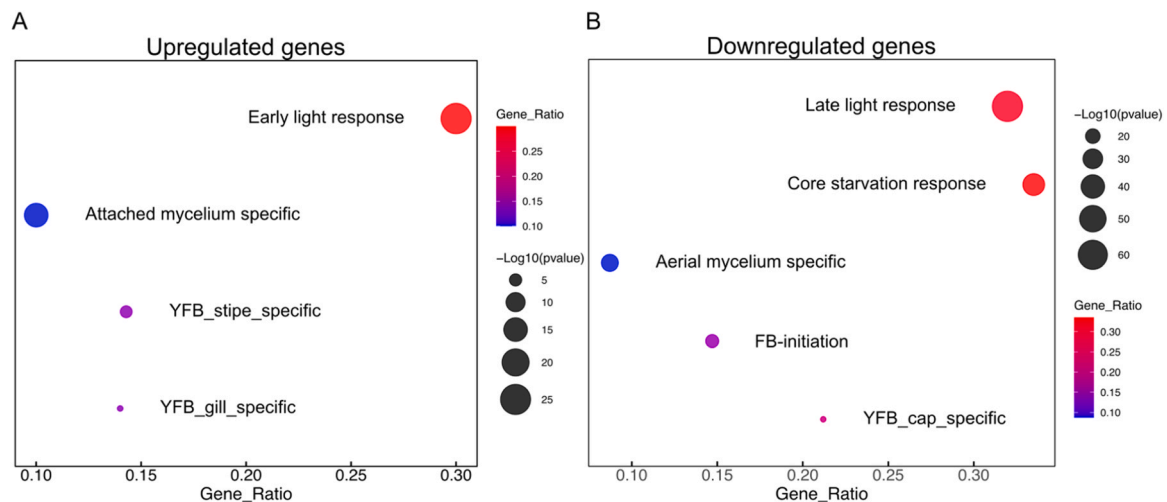


Fig. 8. The custom gene set enrichment analysis of upregulated DEGs (A) and downregulated DEGs (B) with a significance threshold of FDR adjusted p-value (Wald test) < 0.01.

defense; the fungal peptidase inhibitor *pic1* (Sabotić et al., 2012) (367957, $FC_{wt/\Delta gcd1}$: 27.99, MEROPS family I66) and a thaumatin-encoding gene identified as defense-related protein by Plaza et al. (2014) (496344, $FC_{wt/\Delta gcd1}$: 2.74). *Pic1* is a cospin that functions as an effector of a fungal defense mechanism against fungivorous insects, whereas thaumatins are involved in antimicrobial defense against bacteria and fungi (Sabotić et al., 2012). Given the defense-related function of the above two genes, we mined ultra-low input RNA-Seq data (Supplementary Table 3k) and found that these two genes exhibited very high in the universal veil (Supplementary Fig. 7), suggesting a role at the veil surface. The downregulation of this gene aligns with the absence of a universal veil in the $\Delta gcd1$ mutant.

We also found glucose receptor-encoding genes (*Gpr1* in *S. cerevisiae* (Kraakman et al., 1999), that overlap between *gcd1* downregulated DEGs and the cap-specific gene set. Out of five *Gpr1* genes predicted in *C. cinerea*, three are downregulated (426382, $FC_{wt/\Delta gcd1}$: 3.57; 559335, $FC_{wt/\Delta gcd1}$: 2.13; 263790, $FC_{wt/\Delta gcd1}$: 2.17) and one was postulated to be a cap-upregulated gene (Krizsán et al., 2019). *Gpr1* is a G protein-coupled receptor (GPCR) that serves as a primary sensor for extracellular glucose in fungi (Brown et al., 2015; Li and Borkovich, 2006), although there is evidence that it can respond to other molecules too (Kuo et al., 2024). The broad downregulation of glucose receptors in the mutant may signify changes in sensing nutrient availability or other signaling mechanisms.

FB initiation genes were identified by Krizsán et al. (2019) as those whose expression increased at least fourfold at the hyphal knot and stage 1 primordia, relative to vegetative mycelium. Among 585 downregulated DEGs, there are 86 genes (15 %) overlapping with this category (FDR adjusted p-value = 2.91×10^{-18} , Hypergeometric test; Supplementary Table 3), while only 19 upregulated DEGs (5 %) could be classified to this group (FDR adjusted p-value > 0.05, Hypergeometric test). This underscores that the genes downregulated in $\Delta gcd1$ are associated with early developmental processes leading to the emergence of tissue types.

Beyond genes identified by GO and gene set enrichment analyses, we found a notable downregulation of three Pcl1/2-like cyclin homologs (1001791, $FC_{wt/\Delta gcd1}$: 2.93; 1001807, $FC_{wt/\Delta gcd1}$: 2.93; 384611, $FC_{wt/\Delta gcd1}$: 2.71). Cyclins are conserved and generally not expressed dynamically during fruiting body formation (Krizsán et al., 2019). Pcl1/2-like cyclins partner with the cyclin-dependent kinase Pho85 to regulate the cell cycle, polarity and morphogenesis in *S. cerevisiae* (Zou et al., 2009), and affect germination, cell division, and sporulation in filamentous fungi (Campanella et al., 2022; Schier et al., 2001). The role of Pcl1/2 cyclins in fruiting body development is unknown, nevertheless, we

hypothesize they participate by regulating the cell cycle.

3.5.2. Upregulated genes

Upregulated genes are associated with 34 significantly enriched GO terms (Fisher's exact test, p-value ≤ 0.05). Given that many terms were clearly mitochondrion-related, we examined the genes within each term and categorized them as mitochondria-related or other, using subcellular localization predictions for classification (Fig. 6; green vs. other colors). Among these mitochondrion-related GO terms, we found ones referring to organelle parts, mitochondrial ribosomes, and respiration/metabolism, which overall suggest increased mitochondrial activity in the mutant. In other mitochondria-related GO terms, we identified proteins involved in energy production (GO:0006086 “pyruvate decarboxylation to acetyl-CoA”; GO:0006099 “tricarboxylic acid cycle”; GO:0015986 “proton motive force-driven ATP synthesis”).

The GO analysis highlighted several ribosomal terms. To check if these correspond to mitochondrial or cytoplasmic ribosomes, we annotated the entire ribosomal apparatus in *C. cinerea* (Supplementary Table 9a). We found 92 ribosomal genes, 67 mitochondrial ribosomal genes and 42 ribosome biogenesis genes. In the context of the $\Delta gcd1$ upregulated genes, we found a significant overrepresentation of mitochondrial ribosomal genes (DEGs/All annotated, 34/67, Fisher's exact test p-value 7.0×10^{-36}) (Supplementary Table 9b). We also observed three GO terms related to the cytoskeleton. They correspond to four tubulin genes, with two beta-tubulins (393528, $FC_{\Delta gcd1/wt}$: 1.59 and 519006, $FC_{\Delta gcd1/wt}$: 2.18) and two alpha-tubulins (176636, $FC_{\Delta gcd1/wt}$: 1.69 and 359665, $FC_{\Delta gcd1/wt}$: 1.74). Tubulins form the core structural components of microtubules, which are known to mediate organelle positioning and intracellular transport, including mitochondrial dynamics (Westermann and Prokisch, 2002). We hypothesize that tubulin upregulation also corresponds to a higher number of mitochondria and thus increased need for positioning.

Taken together, we interpret the GO results of upregulated genes as a broad signal for increased expression of mitochondria-related genes, which in fungi may reflect increased energy demands and stress responses (Itoh et al., 2020; Loginov and Šebela, 2016; Sephton-Clark et al., 2018). In our case, we hypothesize that this may suggest elevated energy consumption during the hyphal knot stage of the mutant, which may be caused by inadvertent misregulation as a result of the gene knockout in the mutant.

4. Discussion

In this study we described a novel transcription factor, GCD1 that is

required for fruiting body morphogenesis in the model mushroom *Coprinopsis cinerea*. We show that its deletion leads to disturbed morphogenesis and the lack of veil tissues, resulting in a gymnocarpic (open) fruiting body. We showed that GCD1 belongs to the fungal-specific Con7 subfamily of C2H2 zinc-finger transcription factors, which is conserved and harbors a single gene in most Dikarya species, suggesting high functional conservation.

Members of the Con7 subfamily have been primarily investigated in plant pathogenic fungi. These genes have been functionally characterized in *Pyricularia oryzae* (Odenbach et al., 2007), *Fusarium oxysporum* (Ruiz-Roldán et al., 2015), *Parastagonospora nodorum* (Lin et al., 2018), *Fusarium graminearum* (Shin et al., 2024) and two *Colletotrichum* species (Zhou et al., 2024) to date, plant pathogens in which the deletion of the gene yielded reductions in pathogenicity and virulence as well as diverse morphogenetic (e.g. conidiation) defects. A consensus across these studies is that Con7 homologs regulate pathogenicity and morphogenesis; with mechanistic insights suggesting that the defects in the former indirectly result from defects in morphogenesis. In terms of morphogenesis, Con7 homologs have been linked to conidiation, the development of sexual and infection structures (e.g. appressoria), but not vegetative growth (Shin et al., 2024). The latter agrees with our observations in *C. cinerea*, where we found that *gcd1* is hardly expressed in vegetative mycelium and its deletion led to a marginal difference in vegetative growth rate. On the other hand, it is highly expressed in sexual fruiting bodies and we documented a significant impact of its deletion on the latter. Based on these observations we speculate that the regulation of non-vegetative morphogenesis may be a general property of the Con7 subfamily of fungal transcription factors.

We might ask, then, whether the similar role of Con7 homologs across species manifest in shared target genes. $\Delta gcd1$ transcriptomic results and target genes of Con7 homologs in other species show some distinct similarities. Several studies reported that cell wall synthesis and remodeling enzyme genes (e.g. chitinases) are Con7-dependent (Lin et al., 2018; Odenbach et al., 2007; Ruiz-Roldán et al., 2015; Shin et al., 2024). Our RNA-Seq data indicated a downregulation of CE4 chitin deacetylases, GH18 chitinases and multiple glucanases in *C. cinerea*, raising the possibility that these are targets of GCD1, although direct evidence is missing in our case. These similarities suggest that, like Con7 homologs in ascomycetes, GCD1 is a primarily morphogenetic transcription factor in mushroom-forming fungi.

Transcriptome analyses provided insights into the altered gene expression that characterizes the $\Delta gcd1$ phenotype. Downregulated genes included ones related to the fungal cell wall and encoding various chitinases and glucanases, several hydrophobins, kinases (FunK1 and tyrosine kinase-like kinases), cyclins among others. We demonstrated that the GH30.3 family, which encodes glucanases, has undergone an evolutionary expansion in mushroom-forming fungi, suggesting a link to fruiting body development. Upregulated genes, on the other hand, were predominantly related to energy metabolism. We speculate this is linked to the unregulated growth of the mutant, which eventually results in considerably larger primordia than in the wild type. However, as these patterns were detected at the hyphal knot stage, which did not show any morphological differences with wild type strains, the upregulation of mitochondrial genes may be a potentially direct consequence of the deletion of *gcd1*.

GCD1 is currently the single functionally studied member of the Con7 subfamily in the Agaricomycetes. The closest homolog of GCD1 with functional information is ZFC3 in *Filobasidiella (Cryptococcus) neoformans*, however, this has only been examined in the context of pathogenicity (Lee et al., 2020). Future studies should investigate more species to more thoroughly understand its role in fruiting body morphogenesis and beyond. Speculations of similarly important morphogenetic roles in other species are underpinned by the expression dynamics GCD1 homologs of other species comparable to that in *C. cinerea*. Another avenue for future research is investigating the regulatory network of GCD1, both upstream, its regulators, and

downstream, its target genes. As mushroom functional genomics improves, these will come more within reach, even for proteins like GCD1 which appears hard to handle using functional genomics essays.

Taken together, this study demonstrated that GCD1 is required for proper fruiting body morphogenesis in a model basidiomycete. The mutant phenotype, phylogenetic conservation of the protein sequence and the transcriptomic results combined with data on other Con7 homologs suggest that this subfamily of transcription factors is broadly related to morphogenesis in the fungal kingdom. While this assertion will need to be tested in the future, the sequence conservation and function of the Con7 subfamily seems to transcend the > 500 million years of evolution since the Asco- and Basidiomycota diverged from each other.

Author contributions

H.W. and L.G.N. conceived the study. H.W., Z.M., M.V., X-B.L., B.H., Z.H., E.A., A.F., Z.K., and Z.L., performed the wet-lab experiments. H.W., Z.M., B.H. and L.G.N. processed the bioinformatics data. H.W., Z.M., and L.G.N. analyzed the data and prepared figures. H.W., Z.M., and L.G.N. wrote the original manuscript. All authors have read and approved the manuscript.

CRediT authorship contribution statement

Edit Ábrahám: Writing – review & editing. **Anett Fürtön:** Writing – review & editing, Methodology. **Botond Hegedüs:** Writing – review & editing. **Zhihao Hou:** Writing – review & editing. **László G. Nagy:** Writing – review & editing, Supervision, Project administration. **Hongli Wu:** Writing – original draft, Methodology, Formal analysis, Conceptualization. **Zsolt Kristóffy:** Writing – review & editing. **Zoltán Lipinszki:** Writing – review & editing. **Zsolt Merényi:** Writing – review & editing, Methodology. **Máté Virágh:** Writing – review & editing, Methodology. **Xiao-Bin Liu:** Writing – review & editing, Methodology.

Declaration of Competing Interest

The authors declare no competing interests.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.micres.2026.128462.

Data availability

The raw RNA sequencing data have been deposited in the NCBI Sequence Read Archive under BioProject ID PRJNA1353146.

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