Phenotype to genotype in Neurospora crassa: Association of the scumbo phenotype with mutations in the gene encoding ceramide C9-methyltransferase

Erin L. Bredeweg a, Kevin McCluskey b,1, Scott E. Baker a,c,*

a Functional and Systems Biology Group, Environmental Molecular Sciences Division, Pacific Northwest National Laboratory, Richland, WA, USA
b Fungal Genetics Stock Center, Kansas State University, USA
c DOE Joint BioEnergy Institute, Emeryville, CA, USA

* Corresponding author.
E-mail address: scott.baker@pnnl.gov (S.E. Baker).

1 Current affiliation: Bolt Threads, Inc, Emeryville, CA, USA

Using a legacy of genetic mutants of Neurospora crassa, paired with resequencing efforts through JGI, we have identified the gene responsible for the ‘scumbo’ mutant. This early morphological mutant was described as “irregular flat, spreading growth with knobby protrusions and abnormal conidiation, but no free conidia. Mycelium usually appears yellowish rather than orange. Female fertile.” (Perkins, Radford et al. 2000). Our further investigation has found new insights into the identity and associated functions of scumbo as a ceramide C9 methyltransferase, previously annotated as “similar to cyclopropane-fatty-acyl-phospholipid synthase”, encoded by the gene NCU07859. This enzyme performs a fungal-specific methyl modification of glycosyl-ceramides and has implications for membrane homeostasis and hyphal polarity in filamentous fungi.

1. Introduction

Morphology is an easily scored phenotype for filamentous fungi. As such, a significant number of morphological mutants have been identified, genetically mapped, and characterized in the model fungus, Neurospora crassa (Garnjobst and Tatum 1967). When Beadle and Tatum performed screens to understand the biochemical genetics of N. crassa, they also isolated a series of mutants called ‘visible’ (vis), a catchall name for genes with morphological phenotypes (Houlahan, Beadle et al. 1949). Morphological mutants from mutagenesis screens may have defects that result in a disruption to polar hyphal growth and/or branching, cytoskeletal structure or function, or secretory genes (Seiler and Plamann 2003, McCluskey, Wiet et al. 2011, Lara-Rojas, Bartnicki-Garcia et al. 2016). For example, N. crassa smco-9, or semi-colonial-9 was thought to be caused by altered “branching enzyme” expression (Abramsky and Tatum 1976) while alpha-COP coatamers were found to be essential for polarized growth in Aspergillus nidulans (Whittaker, Lunness et al. 1999). Additional determinants of differentiation and morphology may be associated with handling or modifying membrane lipids, such as flipases which were recently characterized in A. nidulans (Schultzhaus, Cunningham et al. 2019), or the effects of phospholipid methyltransferase, as seen in mutants of Pestalotiopsis microspore (Akhberdi, Zhang et al. 2018).

Some of the earliest discovered morphological mutants were used in early studies for genetically locating auxotrophic biochemical genes. One of the vis alleles (5801 - FGSC strain 49) was later mapped and renamed scumbo (sc) (Barratt, Newmeyer et al. 1954). Neurospora crassa morphological mutants are classified into several different groups based on their features (Garnjobst and Tatum 1967). scumbo is classified a semi-colonial mutation, described as a category of “mutants that begin growth on agar as a small colony and sooner or later produce a flare of wild-type-appearing hyphae, with or without conidia.” (Garnjobst and Tatum 1967). The scumbo phenotype mutant is easily recognized by its low spreading growth, knobby protrusions, and abnormal conidiation (Barratt, Newmeyer et al. 1954). Fertile as a female and residing within Linkage Group III, scumbo made an excellent marker that was utilized to genetically map leucineless or leu-1 (Houlahan, Beadle et al. 1949). Analysis by scanning electron microscopy confirmed that the scumbo mutation resulted in “abnormal conidiohores” (Springer and Yanofsky 1989). While multiple studies have shown that morphological mutations could lead to defects in cell wall composition, the nature of many of these genes remained elusive. For example, sc was shown to have
differences in cell wall sugar composition compared to wildtype (Car
demil and Pincheira 1979). A single allele of a modifier of scumbo, mod-sc was isolated and mapped to LGIV (Hsu 1963). A recent screen of Neurospora wild type isolates revealed several morphologies of conidi
ophore development (Krach, Wu et al. 2020) but did not reveal phenomeno
types similar to the extreme scumbo defect.

Phenotypes tracked in morphological mutants also include osmo
sensitivity and resistance to antifungal compounds, such as dicarbox
imides, e.g., vinclozolin. A linkage between these phenotypes has been described for a shared mode of action in which dicarboximides affect osmo
tropic regulation and membrane function which also is regulated through the histidine kinase os-I pathway (Grindle 1984, Schumacher,
Enderlin et al. 1997, Fujimura, Ochiai et al. 2000, Ochiai, Fujimura et al.
2001, Cui, Beever et al. 2002, Oshima, Fujimura et al. 2002). Resistance to vinclozolin results in osmosensitivity. Morphological mutations in N. crassa genes with phenotypes similar to sc have been identified, including the classical morphological mutants, smco-8 and smco-9 (Grindle and Temple 1983).

Despite their utility for genetic mapping, a significant number of morphological mutations in N. crassa remain “anonymous” or unasso
ciated with a physical locus in the genome. The use of next generation sequencing technologies to rapidly identify mutations and associate them with phenotypes has been called “fast forward genetics” (Darby and Hall 2008, Schneeberger and Weigel 2011). This strategy has great potential to link genes with biological function in fungal systems (Baker 2009, Baker and Bredeweg 2016, Baker 2018). In previous work we used genetic map data combined with a population of single nucleotide var
iants from several N. crassa strains to identify candidate mutations to associate with anonymous mutant genes (McCluskey, Wiest et al. 2011). Another approach is to sequence the genome of multiple strains that carry independently isolated alleles of the target mutant gene that have been shown to be alleles by complementation analysis in genetic crosses (Garnjobst and Tatum 1967, Perkins, Radford et al. 2000). These different alleles may contain different mutations in a single gene. In this study we describe our use genome resequencing to facilitate the rapid identification of the morphological mutants of N. crassa, sc as well as phenotypically related mutants, smco-8 and smco-9. Identification of the causal gene for the scumbo phenotype by sequencing allowed us to test the hypothesis that NCU07859 is scumbo by gene knockout. Testing and con
firmation of historically collected mutants merges longstanding ob
servations with gene function, and morphological structures and func
tional inter-dependencies.

2. Materials and Methods

2.1. Genomic DNA preparation and sequencing

Strains of N. crassa were maintained and DNA extracted as described previously (Gabriel, Thieme et al. 2021). To summarize, N. crassa strains shown in Table 1, were preserved on anhydrous silica gel (Perkins 1962) in the Fungal Genetics Stock Center collection with minimal cycles of preservation and regrowth, some for as long as 50 years without pas
sage. Genotypes were routinely tested as part of best practices (Wiest, Schnittker et al. 2012). Cultures for DNA extraction were prepared by first sprinkling a few grains of silica gel containing preserved conidia onto agar solidified Vogel’s minimal medium (Vogels 1956). After approximately one week, conidia were inoculated into 10 ml Vogel’s minimal medium in a 50 ml disposable conical test tube using a heat sterilized inoculating loop. These cultures were incubated at room temperature (18 – 22 °C) with shaking at approximately 100 cycles per minute for 2 to 3 days. A pad of mycelia was removed from the culture with a sterile wooden applicator stick and blotted dry on sterilized paper towels. 90 – 120 mg of tissue was weighed and transferred to a macer
ating tube and DNA extraction was carried out according to the in
structions provided by the manufacturer (ZR Fungal/Bacterial DNA MiniPrep, Zymo Research, Irvine, CA). Tissue maceration was carried out using a Mini-Beadbeater-16 (BioSpec Products, Bartlesville, OK) for 30 seconds and set to half of maximum power. DNA quality was assessed by separating a sample in an agarose gel using standard practices and by spectrophotometric analysis using a NanoDrop microvolume spectro
photometer (Thermo Scientific, Wilmington, DE). DNA was held at -20°
until being sent on dry ice to the US DOE JGI for sequencing and analysis as previously described (McCluskey, Wiest et al. 2011, Reilly, Kim et al. 2018).

Briefly, the JGI generated DNA libraries for paired-end sequencing method using MiSeq 2 × 150 bp (~30 × coverage) or HiSeq 2 × 100 bp (~100 × coverage). Genome sequences from each strain were compared to the reference genome for identification of SNPs and indels using BCFTools and GATK tools (McKenna, Hanna et al. 2010, Li 2011) and subsequently compared against each other to identify mutations unique to each strain compared against an aggregate of strain resequencing data from the Neurospora resequencing project (doi: 10.25585/1487571).

2.2. Isolation and characterization of homokaryon deletion strains

FGSC 13992 (NCU07859 heterokaryon) was used as the maternal strain with FGSC 2489, 74-OR23-1VA as the fertilizing strain in a cross. Resulting ascospores were isolated and tested for hygromycin resistance, the marker used to delete genes in the deletion collection. To test the genotype of the isolated ascospore homokaryons, strains were grown on hygromycin B (100 μg/ml) in comparison to wild type and the classic scumbo mutant FGSC 49. Inoculum for these tests was generated by growing test strains on Vogel’s medium agar. Uniform agar blocks (3mm square) of hyphae were cut from the colony edge and placed on plates (6 cm) containing 8 ml of Vogel’s glucose agar alone or containing drug as indicated. Imaging was after 1 week of strain outgrowth.

2.3. Osmotic challenge plate assay

Conidia or hyphal fragments were cultivated on agar solidified Vogel’s minimal medium with 1.5% glucose for 3 hours. Uniform blocks (3 millimeters per side) containing hyphae were cut and placed...
on new small plates (6 cm) containing 6 mL the same medium with or without the osmotic agent listed (.25 M NaCl, 1 M NaCl, 3% Ethanol). Imaging was conducted after 120 hours of growth at room temperature.

2.4. Strain generation and microscopy for GFP localization of ceramide C9-methyltransferase

To generate a C-terminal transcriptional fusion to observe localization of the scumbo enzyme, we designed primers to amplify 1 kb regions up and downstream of the stop codon of NCU07859 using q5 polymerase (New England Biolabs) reaction conditions (5’ fragment from OEB 392, 5’-GACCCCAAACAGAGGAGGAGA-3’ and OEB393, 5’-CCTCGGGCTCCCG CCTCGCGGCGCTCCGGGTTGAGGACAGTGGG-3’; 3’ fragment from OEB394, 5’-TGCTATACGAAAGTTATGGATCCGAGCTCGAAGCG GCAAAGGAGGACA-3’ and OEB395, 5’-CCATTAAGGATTCGAGGCAGGACA-3’). These 5’ and 3’ fragments were integrated by overlap PCR with a plasmid fragment (pGFP∷hph∷loxP) containing GFP and hph (amplified from the plasmid with primers OEB59, 5’-CGAGCTCGGATCCATTAAGGATTCGAGGCAGGACA-3’ and OKP31, 5’-GGCGGAGGCGGCGGAGGCGGAGGAGAAGAAGGCAGGAGGAG-3’). We used primers to create an overlap in the middle of hph (OEB57, 5’-GTGCTTTCAGCTTCGATGTAGG-3’; paired with OEB392; and OEB58, 5’-AGAAGATGTTGGCGACCTCG-3’ paired with OEB395), while leaving individual 5’ and 3’ PCR fragments with an incomplete hph gene to ensure homologous recombination (Honda and Selker 2009). Initial fragments were amplified using 32 cycles, followed by gel purification. Overlap PCRs were run 5 cycles with only template DNA fragments (5’ fragment or 3’ fragment each with GFP-hph fragment), followed by addition of primers and a further 32 cycles. These
PCR fragments were gel purified and added in equimolar amounts to *Neurospora crassa* conidia (FGSC9718, delta mus-51::bar+, mat a) for electroporation (Colot, Park et al. 2006). Details of PCR primer placement and overlap construction are included in Supplemental Fig. SB. Colonies were picked to individual slants, and screened for GFP signal by fluorescence microscopy as noted below. A GFP-positive strain from this transformation TEB146.1 was crossed to the wild type strain 74-OR23-1VA (FGSC2489, mat A) to obtain the homokaryon strain FEB369.

Conidia of strain FEB369 were grown on Vogel’s agar in a 100 mL Erlenmeyer flask, with 5 minutes of sunlight exposure at 24 and 28 hours to support conidiation before collection at 7 days. Conidia were collected and stored in sterile distilled water at 5°C. For confocal microscopy, conidia were extensively diluted, and grown in 2 mL of 1% glucose Vogel’s liquid media in 35 mm dishes, with amendments. Amendments include 0.25 M NaCl or 3% ethanol. For imaging, a small section of dispersed tissue was transferred onto a glass slide in 5-10 μl of media and covered for imaging with a glass coverslip. Imaging was performed on the Leica 710 confocal laser scanning microscope with a 100X oil immersion lens.

2.5. Conidia germination microscopy

Conidia grown as above were collected by wetted wooden stick or from powdery tissue released on plate lid and suspended in sterile distilled water. A 50 μl aliquot of this suspension was added to 150 μl 2% Vogel’s liquid medium in a 1.7 mL Eppendorf and incubated without shaking at room temperature for 6-7 hours. Results of this experiment are presented in Supplemental figure SA.
knockouts for NCU07589 were deposited as heterokaryons. We under-
progeny from each mating type were isolated. The morphological 
were obtained from the 
methyltransferase domain. Allele R2386 contains a frameshift while 
phenotype of homokaryons derived from the deletion collection strain 
used to delete genes in the deletion collection. Multiple ascospore 
spores were isolated and tested for hygromycin resistance, the marker 
took the isolation of homokaryons using a genetic cross. FGSC 13992 
was an important allele of 
isolement (Table 1). This gene has also been annotated as 
cyclopropane fatty acyl phospholipid synthase, and as gel-9 (FungiDB). 
This enzyme, only found in fungi, adds a methyl-group to the C9 carbon 
of a ceramide hydrocarbon chain. The first mutant allele of 
scumbo 
isolement, 5801, contained a six bp deletion causing a change at glutamic 
acid-histidine-valine (EHV) to a single aspartic acid (D) at residue 341. 
These residues are conserved across fungal orthologues and occur in a 
methyltransferase domain. Allele R2386 contains a frameshift while allele R2503 has a mutation in the intron splice site acceptor (Fig. 1).

After identification of the locus consistent with the scumbo phenotype, 
strains carrying targeted gene deletion mutations at NCU07589 were 
obtained from the Neurospora gene deletion collection (Colot, Park et al. 2006, Dunlap, Borkovich et al. 2007, Park, Colot et al. 2011). The 
knockouts for NCU07589 were deposited as heterokaryons. We under-
took the isolation of homokaryons using a genetic cross. FGSC 13992 
(NCU07589 heterokaryon) was used as the maternal strain with FGSC 2489, 74-OR23-1VA as the fertilizing strain in a cross. Resulting asco-
spores were isolated and tested for hygromycin resistance, the marker 
used to delete genes in the deletion collection. Multiple ascospore 
progeny from each mating type were isolated. The morphological 
phenotype of homokaryons derived from the deletion collection strain 
mirrors that of the classical scumbo allele containing strains (Fig. 2).

While identifying scumbo-associated genes and morphological mu-
tants, we also re-sequenced strains for smco-8, smco-9, and a modifier of the scumbo defect, mod(sc) as part of a larger resequencing project (doi: 10.25585/1487571). (Table 1). We identified mutations in ADP- 
ribosylation factor GTPase activating protein (ARF GAP) (NCU08811), 
serine/threonine-protein kinase gad8 (NCU07280) and coatomer beta 
subunit (NCU04404), respectively.

3.2. Phenotyping

In many fungi, C9 methylation of ceramide is a key step in fungal 
synthesis of glucosylceramide and galactosylerceramide and is implicated 
in pathogenicity [in Cryptococcus neoformans (Singh, Wang et al. 2012, 
Raj, Nazemidashtarjandi et al. 2017), Pichia pastoris (Ternes, Sperling et al. 2006), and Fusarium graminearum (Ramamoorthy, Cahoon et al. 2009)], implicated in pH stress response and linear growth, [in Asper-
gillus nidulans (Levery, Momany et al. 2002) and Candida albicans (Oura and Kajiwara 2010)]. It has also been implicated in cell differentiation, 
with ceramide monohexosides (CMHs) containing this modification 
being found in hyphal, but not conidial tissue of 
C9-methyltransferase and its product play in the development of plasma 
membrane we tested wildtype and mutant strain response to a variety of 
osmotic stresses, which are known to be dependent on plasma mem-
brane stability (e.g. (Ianutsevich, Danilova et al. 2016)) (Fig. 3). Our 

tests indicated susceptibility of scumbo to hyphal extension under os- 

motic stressors of ethanol and salt.

3.3. Localization of ceramide C9-methyltransferase by microscopy

We sought to explore the localization of ceramide C9-
methyltransferase in N. crassa and constructed a C-terminal GFP tagged 

ceramide C9-methyltransferase strain. Localization of this enzyme has not been previously shown directly in filamentous fungi. After 
transformation with a C-terminal GFP cassette in a NHEJ-defective 
strain (FGSC9718, Δmus-51::bar+; mat a), the GFP positive (screened 
by microscopy) strain was backcrossed to 74-OR23-1VA (XEB 37). The 
resulting ascospores were heat shocked, picked to individual agar slants, 
and screened for GFP. The resulting strain (XEB 37.1, FEB369) was 

morphologically indistinguishable from wildtype indicating that the 
addition of GFP did not noticeably alter enzyme function. Interestingly, 
the localization of the GFP signal changes over time, with respect to the 
hyphal tip, and osmotic stressor (Figs. 4, 5). NCU07859 contains 2 
transmembrane domains (amino acids 53-75, and 87-109) allowing 
membrane association.

4. Discussion

A prodigious number of classical genetic mutants remain “anony-
mous”; the low cost and high coverage generated by current sequencing
methods makes it tractable to simply re-sequence these strains to identify the mutation responsible. Indeed, resequencing *N. crassa* has been particularly successful, associating genes with mutant phenotypes. Here, we describe the identification of NCU07859 which encodes a ceramide C9-methyltransferase as the classical *Neurospora crassa* morphological mutant, *scumbo*. Supporting this identification is the fact that multiple alleles of *scumbo* were sequenced and each had mutations in NCU07859. In addition, we generated homokaryotic strains from the *N. crassa* gene deletion project heterokaryon strain. These strains with deleted NCU07859 phenocopied the classical *scumbo* strains. The strain from the *N. crassa* gene deletion project deposited at the Fungal Genetics Stock Center is a heterokaryon which led to the hypothesis that NCU07859 is an essential gene in *N. crassa*. Detailed phenotypic analysis was done on the heterokaryon by Huber et al (Huber, Oemer et al. 2019). Research from *Fusarium* and *Aspergillus nidulans* have concluded that ceramide C9-methyltransferase is essential. However, in some yeasts where the gene encoding the ceramide C9-methyltransferase has been deleted growth is impacted but not eliminated. By crossing the *N. crassa* heterokaryon with wildtype we generated homokaryotic mutants which are morphologically identical to classically genetically derived *scumbo* strains. The existence of homokaryotic *scumbo* classical mutants and generation of homokaryotic deletion strains indicate that NCU07859 is not an essential gene.

Sensing of osmotic stress occurs at the cell membrane and initiates a significant cell signaling response. Moreover, the cell plasma membrane composition is remodeled at both the lipid and protein levels. In *Neurospora*, the two-component histidine kinase *nik1*(os-1)-*rrg1* responds to osmotic stress triggering a downstream response through the *hog1* MAP kinase cascade (os-2, os-4, os-5)(Jones, Greer-Phillips et al. 2007). Evidence from a variety of species across the fungal kingdom indicates that the histidine kinase sensors are organized by lipid rafts and that sphingolipids play an important role in their formation (Singh, Wang et al. 2012, Tanigawa, Kihara et al. 2012, Fernandes, de Castro et al. 2016, Raj, Nazemidashtarjandi et al. 2017). Yeast and filamentous fungal osmosensor sensor and signaling machinery are dependent upon glucosylceramides for proper function. We hypothesize that the sc phenotype is due in part to disruption of these rafts caused by disruption to the glucosylceramide biosynthetic pathway.

Fig. 5. *Scumbo-GFP in a time-series*. Images were taken of the same hyphae of Scumbo-GFP, showing accumulation at the end of the hyphae’s Spitzenkörper, or collection of vesicles at the hyphal tip. Scumbo signal fluctuates along the endomembrane system. Each timepoint represents a capture over approximately 2 minutes by scanning confocal microscope. Scale bar is 10 μm in t8.
As cells remodel to deal with osmotic stress, anterograde and retrograde vesicle transport pathways play an obvious role, and both are strongly influenced by sphingolipids. The similar phenotypes of vinclozolin resistance and osmotic sensitivity of sc, smco-8 and smco-9 imply that proteins encoded by these genes may be involved in the biological processes involved in both osmotic stress sensitivity and dicarboximide resistance. Our results indicate that smco-8 (NCU08811) encodes an ADP-ribosylation factor GTPase-activating protein (Arf1GAP) orthologous to S. cerevisiae GLO3 and mod(sc) (NCU04404) encodes a coatomer-beta orthologous to S. cerevisiae SEC26. Multiple studies in Saccharomyces have linked GLO3 and SEC26 (DeRegis, Rahil et al. 2008, Schindler, Rodrigues et al. 2009). Arf1GAPs are linked to COP(I) coatomer vesicle formation during priming and assembly of the coat protein on a membrane surface (Beck, Ruvet et al. 2009). The protein encoded by smco-9 (NCU07280 also called stk-50) is an ACG kinase that is activated by TOR kinase, with homologs in S. cerevisiae (YPK1) (Roelants, Torrance et al. 2002) and S. pombe (Gad8) (Martin, Portantier et al. 2017). YPK1 activity is required for plasma membrane lipid and protein homeostasis, cell wall integrity, and endocytosis suggesting a common mechanism for colonial defect formation in the fundamental structure membranes of these colonial phenotypes.

We localized SC to the endomembrane system and the Spitzenkörper in growing hyphae. This localization is consistent with the hypothesis that the plasma membrane is dynamic and characterized by the ability to rapidly remodel in response to stress. The localization phenotype is also consistent with proteins modifying lipids at the golgi apparatus and associated organelle membrane structures. Our observations were done early in growth (i.e. within 20 hours of germination) (Figs. 4, 5, and SB). Additional expression patterns are suggested for older tissue: the gene is expressed and protein accumulates at the cell periphery where modifications take place (Rasuga and Glass 2008).

Plants and animals do not have orthologs of the ceramide C9-methyltransferase making C9-methylation a unique modification which distinguishes fungal sphingolipids from plant and animal lipids (Temes, Sperling et al. 2006). The presence of C9-methylation has also been used in defenses from other organisms, such as plant and insect defenses against P. pastoris and C. albicans (Thevissen, Warnecke et al. 2004) but is less important in F. graminearum susceptibility (Ramamoorthy, Cahoon et al. 2009). The N. crassa heterokaryon for NCU07859 does show an altered lipid profile and was tested for anti-microbial protein susceptibility (Huber, Oemer et al. 2019). It is unknown if the scumbo deletion homokaryon would perform differently. Yeast pleiotropic drug resistance pathways and regulation have also shown linkage to levels of sphingolipids and biosynthetic enzyme expression (Hallstrom, Lambert et al. 2001, Kolaczkowski, Kolaczkowska et al. 2004). Taken together this modification is a gateway to fungal-specific drug development and structural regulation of stress responses.

Mutants at the locus NCU07859 have a phenotype classically described as poor conidial development. Our observations during osmotic challenge plate assays indicated formation of powdery conidia-containing tissue, which suggests that scumbo doesn’t inhibit conidial development under all conditions. For example, conidiation was associated with physical disruption of hyphae, as occurred during agar block cutting. Conidial development was also not observed under some stress conditions (e.g., EtOH stress, Fig. 3). Though functional sphingolipid C9-methyltransferase activity may be important for conidial germination or subsequent processes, we did not observe any defects in the ability of scumbo conidia to begin hyphal elongation. Other studies have noted NCU07859 is strongly upregulated during progression through the four stages conidial germination (Wang, Miguel-Rojas et al. 2019).

Extensive data on Neurospora population genetics and transcriptomics are available at FungiDB (Stajich, Harris et al. 2012, Baseño, Pulman et al. 2018). These data show that there is only one transcript from NCU07859 and that there are no non-synonymous or nonsense mutations at this locus. This strain population includes reference strains, mutants, and a group of 48 strains from wild populations (Ellison, Hall et al. 2011). Expression of NCU07859 was characterized and is shown to vary during sexual development (Coradetti, Craig et al. 2012, Wang, Lopez-Giraldez et al. 2014), during growth on cellulose versus sucrose (Coradetti, Craig et al. 2012), and among strains from the wild type population (Ellison, Hall et al. 2011).

In sum, identification of scumbo as ceramide C9-methyltransferase emphasizes the importance of lipid membranes in stress resistance, signaling and morphology. The striking morphology and phenotypes of the scumbo mutant suggests that this uniquely fungal lipid modification affects cell physiology and gene expression. Transcript level changes stemming from altered levels of modified glycosylerceramide will be a topic for future work.

CRediT authorship contribution statement

Erin L. Bredeweg: Conceptualization, Writing – original draft, Investigation. Kevin McCluskey: Conceptualization, Investigation, Writing – review & editing. Scott E. Baker: Conceptualization, Project administration, Writing – original draft, Investigation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.hal.2021.102169.

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