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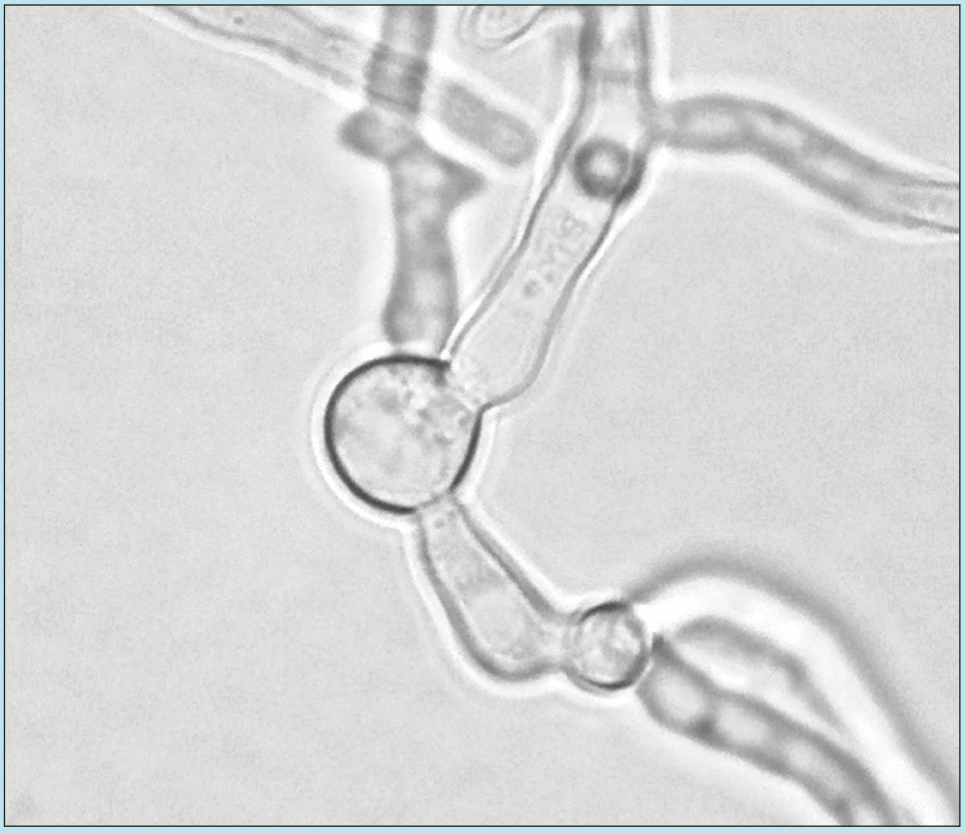

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Cover Photograph: Microscopic image of a swollen fungal spore due to reduced expression of COG5. Photograph © Sara Gremillion.

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Conserved Oligomeric Golgi Complex Subunit 5 Contributes to Fungal Growth

Sara Gremillion[1*], Erin Berube[2], Nick Brodak[2], and Ansley Osborn[2]

Abstract - The Conserved Oligomeric Golgi (COG) complex is a tethering complex involved in retrograde transport of Golgi vesicles. In yeast, the COG complex has 8 subunits arranged into Lobe A (COG1–4) and Lobe B (COG5–8). Lobe A subunits are critical for function of the complex and for the viability of cells while those in Lobe B are not. In the filamentous fungus *Aspergillus nidulans* (Eidam) Winter, Lobe A has been linked to proper polarized growth, but there is no information on the role of Lobe B in filamentous growth. Through a promoter replacement strategy, under-expression of the Lobe B subunit COG5 led to abnormal growth, providing evidence that, unlike yeast, Lobe B of the COG complex is necessary for growth.

Introduction. Fungi are eukaryotic organisms noted for their variety of excreted molecules such as antibiotic compounds, degradative enzymes, and deadly mycotoxins, to name a few (Alexopoulos et al. 1996). At the core of secretion in fungal cells is the ever-active exocytic pathway, a cellular process that involves the creation, organization, and movement of content-carrying vesicles from the endoplasmic reticulum through the Golgi complex to the cell's plasma membrane (Pantazopoulou 2016). The constant departure of lipids and proteins in exocytic vesicles depletes organelles like the endoplasmic reticulum and Golgi apparatus. In order to recycle vesicular components lost by outgoing vesicles, the cell moves incoming vesicles from the plasma membrane to the endosome via endocytosis. Vesicles are then moved from the endosome to the Golgi and then to the endoplasmic reticulum in a process called retrograde transport (Bonifacino and Rojas 2006, Maxfield and McGraw 2004). Retrograde transport within the Golgi complex is of particular importance, as vesicles traveling from trans to cis Golgi cisterna have been shown not only to replenish but also to reorganize Golgi resident proteins and lipids, ensuring correct Golgi function (Glick, et al. 1997, Opat et al. 2001).

Multiple proteins involved in retrograde transport in the Golgi have been identified and include coat proteins (Glick and Malhotra 1998), Rab GTPases (Benli et al. 1996, Martinez et al. 1994), SNAREs (Soluble NSF Attachment Protein Receptor; Malsam and Söllner 2011), as well as golgins and tethering complexes (Witkos and Lowe 2017). The Conserved Oligomeric Golgi (COG) complex is an 8-subunit tethering complex that is an important part of intra-Golgi retrograde vesicle transport (Miller and Ungar 2012, Reynders et al. 2011, Willett et al. 2013). The COG complex is organized into two distinct lobes. In mammalian models, COG2–4 form Lobe A, COG5–7 form Lobe B, and COG1 and COG8 form a bridge between the two lobes (Fotso et al. 2005, Ungar et al. 2005). In humans, mutations in subunits of both lobes are linked to glycosylation-associated congenital disorders, with mutations in Lobe A causing more severe phenotypes (Bruinsma et al. 2004, Kingsley et al. 1986, Miller and Ungar 2012, Oka et al. 2005, Pokrovskaya et al. 2011, Shestakova et al. 2006).

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In fungi, the COG complex has been well-studied in the yeast *Saccharomyces cerevisiae* Meyen ex. E.C. Hansen (Brewer's Yeast), where it was found to be organized similarly to the human complex, with Lobe A consisting of subunits COG1–4 and Lobe B consisting of COG5–8 (Fotso et al. 2005, Ha et al. 2016). Studies found that the subunits of Lobe A play a more critical role in the function of the complex when compared to those in Lobe B. For example, mutated or reduced Lobe A subunit production was linked to Golgi vesicle accumulation, abnormal cisternal shape, mis-glycosylation of proteins, and poor cell growth (Lees et al. 2010, Whyte and Munro 2001). These phenotypes were reduced or absent when subunits in Lobe B are negatively affected.

While the role of the COG complex has been elucidated in yeast, few studies have examined the role of the complex in the filamentous fungi that, unlike yeasts, spend a majority of their lives in a state of continued polarized growth. Beginning life as a single-celled spore, the filamentous fungal spore germinates and swells isotropically. Typically, after one round of mitosis, a single point of polarity is established, and the spore switches to polarized growth of a single germ tube (Momany and Taylor 2000). Over time the germ tube branches, forming new cells called hyphae that also grow in a polarized manner. Fungal spores can establish multiple germ tubes, resulting in a fungal colony with multiple points of active polarized growth (Griffin 1994). Supporting this early growth is the Golgi apparatus. Golgi-derived exocytic vesicles containing the proteins, lipids, and cell wall materials needed for isotropic growth are recruited to all areas of the cell's plasma membrane, then they are relocated to a single point along the plasma membrane in the switch to polarized growth (Bartnicki-Garcia 2002).

While the Golgi apparatus plays a crucial role in filamentous fungal growth, few studies have linked Golgi tethering proteins to polarized growth. In the filamentous fungus *Aspergillus nidulans* (Eidam) Winter, mutations in the subunits COG2 and COG4 (subunits of Lobe A) were associated with altered protein glycosylation, a lack of cell wall integrity, and abnormal polarized growth (Gremillion et al. 2014). Specifically, during spore germination, the COG2 mutation led to over-swelling of spores (extended isotropic growth) and an absence of germ tube formation, while the COG4 mutation led to a range of phenotypes including swollen and misshaped spores and, when germination did occur, abnormally wide germ tubes that often did not maintain polarized growth. A reduced expression of the COG2 and COG4 subunits resulted in germinated spores with abnormal polarization (Gremillion et al. 2014). Building upon the study of the two COG subunits in Lobe A, the current study aims to determine if Lobe B of the COG complex plays a role in filamentous fungal growth. The subunit COG5 was selected as a representative subunit of Lobe B because it has been noted as important for polarized growth of animal flagella (Farkas et al. 2003). The promoter of the cog5 gene in *A. nidulans* was replaced with a regulatable promoter, *alcA*, to create a new strain called *alcA*(p)::COG5. The *alcA* promoter reduces protein expression when grown on media with glucose as the sole carbon source, and it produces wild type protein expression on glycerol-based media. When spores from the *alcA*(p)::COG5 and wild type strains were grown in the two types of media, all spores germinated and produced one or more germ tubes via polarized growth regardless of the growth medium (Fig. 1). Polarity of germ tube development did not appear to be affected by the reduction in the COG5 protein. However, the isotropic growth of the spore appeared to be greater than normal (Fig. 1D). A two-way ANOVA on spore width, a measure of isotropic growth, with Strain (*alcA*(p)::COG5, wild type A1145) and Media Type (glucose, glycerol) revealed a main effect of Media Type, $F(1, 196) = 25.46$, $P < 0.001$. The main effect of Strain was not significant: $F(1, 196) = 0.01$, $P = 0.92$. The Strain x Media Type interaction was significant: $F(1, 196) = 50.01$, $P < 0.001$. The

interaction resulted from a difference between Media Types for the *alcA*(p)::COG5 strain (independent samples *t*-test, t (112) = 9.75, $P < 0.001$). Spores of *alcA*(p)::COG5 grown on suppressive minimal media were significantly wider than that of spores of the same strain grown on inducive minimal media (Table 1). No significant difference between the two media types for wild type was found (independent samples *t*-test, t (84) = 1.27, $P = 0.21$) (Table 1).

In *A. nidulans*, the Lobe A subunits COG2 and COG4 are required for normal growth (Gremillion et al. 2014). Lobe A is also noted as critical for the function of yeast cells while subunits in Lobe B are not (Whyte and Munro 2001). The current study provides evidence that Lobe B is critical for normal growth as the subunit COG5 (part of Lobe B) was identified as important for maintaining proper filamentous fungal growth. The abnormally wide spores observed when COG5 production was reduced indicates the inability of the spores to stop isotropic growth and focus solely on polarized growth of the germ tube or germ tubes. It is possible that the reduced expression of the COG5 protein compromised the function or structure of the COG complex and, by extension, negatively impacted retrograde transport

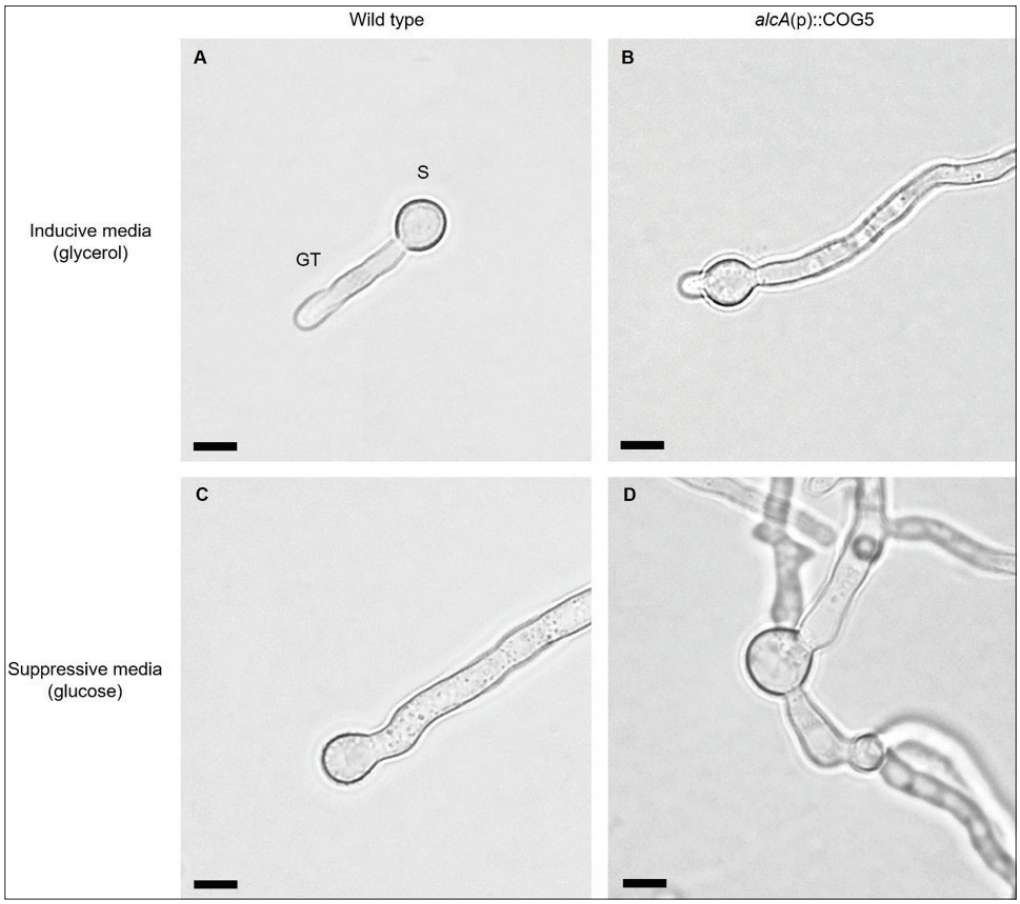

Figure 1. COG5 subunit is required for normal fungal growth. A) Wild type strain, A1145, grown on 1% glycerol inducive minimal media (MM). S = spore, GT = germ tube; B) *alcA*(p)::COG5 grown on 1% glycerol inducive MM; C) Wild type grown on 1% glucose suppressive MM; and D) *alcA*(p)::COG5 grown on 1% glucose suppressive MM. All spores were grown in liquid media at 28C for approximately 17–24 h. Scale bars = 5.0 µm.

in the Golgi. A compromised Golgi apparatus may not be able to correctly create or direct the vesicles needed for proper filamentous growth (Bartnicki-Garcia 2002, Gremillion et al. 2014, Taheri-Talesh et al. 2008). The link between polarized growth and the COG Lobe B in *A. nidulans* mimics what has been found in other organisms that exhibit directional growth. In the plant *Arabidopsis* spp. (Rockcress), wild type pollen tube polarization is negatively affected when subunits COG6 or COG8 (both of Lobe B) are mutated (Rui et al. 2020, Tan et al. 2016). In the animal *Drosophila* spp. (Fruit Flies), the polarized elongation of the sperm tail is disrupted when cells contain a mutation in COG5 (Farkas et al. 2003).

Beyond cell growth, the COG complex has been linked to maintenance of cell wall structure in *Arabidopsis* spp. (Rui et al. 2020, Tan et al. 2016) and proper glycosylation in animals (Farkas et al. 2003, Ungar et al. 2002). In the fungi, proper cell wall structure and protein glycosylation are associated with the COG complex in Brewer's Yeast (Bruinsma et al. 2004, Kingsley et al. 1986, Oka et al. 2005, Pokrovskaya et al. 2011, Shestakova et al. 2006) and in subunits COG2 and COG4 in the filamentous fungus *A. nidulans* (Gremillion et al. 2014). Future studies will focus on the effects of down-regulation of COG5 and the remaining COG subunits of *A. nidulans* on filamentous growth, cell wall structure, and protein glycosylation.

Fungal strains, growth media formulations, and culturing methods. To culture *A. nidulans* strains, a glucose-based complete medium consisting of 1% glucose, 0.2% peptone, 0.1% yeast extract, 0.1% casamino acids, 5% nitrate salts, 1% trace elements, 0.1% vitamin mix, and 1.2 mM L-arginine was used (Hill and Kafer 2001, Hill et al. 2006, Kafer 1977). Minimal media (MM) consisted of 1% glucose or 1% glycerol as the sole carbon source, as well as 5% nitrate salts, 1% trace elements, 0.001% thiamine hydrochloride, and 25 ppb biotin. A glycerol-based minimal medium was also used, which contained all ingredients listed above but replaced 1% glucose with 1% glycerol. When necessary, the media were supplemented with 10 mM uracil and 5 mM uridine and 0.1 mg/ml of riboflavin. Solid media contained 1.5% agar, and 50 mg/ml ampicillin to prevent bacterial contamination.

Creation of the regulatable alcA*(p)*::COG5 strain and testing the effects of the reduced expression of COG5. The *A. nidulans* Fungal Genetics Stock Center strain, A1145, served as the wild type in a reduced expression study on COG5 (McCluskey 2003). The native promoter of the *A. nidulans* cog5 gene, AN1774 (Gremillion et al. 2014), was replaced with the regulatable *alcA* promoter so that protein expression could be controlled based on the available carbon source in the growth medium (Romero et al. 2003). Reduced production of the COG5 protein was achieved on *alcA* "suppressive" MM containing 1% glucose as the primary carbon source. The "inducive" MM, which contained 1% glycerol as the primary carbon source, served as the control as it activates the *alcA* promoter and induces protein production (Romero et al. 2003).

Table 1. Mean spore width (μm) significantly increases when the expression of the COG5 subunit is suppressed in the *A. nidulans* strain *alcA*(p)::COG5 compared to the wild type.[a]

Strain[a]	Mean Spore Width (μm) on Inducive Media (1% Glycerol)	Mean Spore Width (μm) on Suppressive Media (1% Glucose)
alcA(p)::COG5	3.51 ± 0.60	5.12 ± 1.00*
Wild type	4.43 ± 0.80	4.17 ± 0.99

[a]Each strain was grown separately in coverslip cultures containing liquid 1% glucose suppressive minimal media (MM) and 1% glycerol inducive MM, incubated between 17–24 h at 28C. Mean spore width is an average of at least 29 measurements. Standard deviations are indicated after each mean. An asterisk (*) indicates mean spore width values within the same strain that are significantly different ($P \leq 0.001$).

Promoter replacement began with the amplification of two regions from the genomic DNA of A1145 using Phusion High Fidelity PCR Master Mix with HF buffer and accompanying protocol (New England Biolabs, Massachusetts, USA). The "upstream" primers were used to amplify approximately 500 base pairs upstream of the start codon of the *A. nidulans* cog5 gene, and the "gene" primers were used to amplify approximately 500 base pairs within the gene, downstream from the beginning of the start codon (Table 2). The *AfpyrG::alcA*(p) promoter cassette was amplified from pDL141 plasmid (Gremillion et al. 2014) using the "cassette" primers (Table 2). The three PCR products were cleaned using the GenElute™ PCR Clean-Up Kit (Sigma-Aldrich, Missouri, USA), then combined to create a single linear piece of DNA in a fusion PCR reaction (Szewczyk et al. 2006) using Phusion High Fidelity PCR Master Mix with HF buffer and the "fusion" primers (Table 2). The newly fused *alcA*(p)::COG5 DNA product was then transformed into A1145 as described by Osmani et al. (2006), creating a new strain called *AfpyrG::alcA*(p)::COG5, or *alcA*(p)::COG5. The *AfpyrG* gene of the cassette served as the selective marker to screen transformed wild type cells that are deficient in the two pyrimidines uridine and uracil.

To determine if the large fusion DNA product was inserted into the genome of *alcA*(p)::COG5, confirmation primers were used in a PCR using Phusion High Fidelity PCR Master Mix with HF buffer and accompanying protocol with an annealing temperature of 57C (Table 2). The primer set amplifies approximately 200 base pairs upstream and downstream of the expected insertion location, resulting in an expected amplicon of 5.0 kb (Table 2). The primers set was also tested on DNA from the wild type strain, A1145, to serve as a control. The expected amplicon size of genomic DNA without the insertion is 2.32 kb. A 0.65% gel run for 1 hour at 60 mV confirmed the successful insertion of the fusion DNA product.

The effect of the reduced expression of COG5 on fungal growth was observed using coverslip cultures as described by Harris et al. (1994). Spores of the new *alcA*(p)::COG5 strain and the wild type strain were grown at a concentration of approximately 3000 spores/

Table 2. PCR primers used in this study.

Primer[a]	Sequence[b]
*Afpyr*G::*alcA*(p) cassette F[c]	CTGTCTGAGAGGAGGCACTGA
*Afpyr*G::*alcA*(p) cassette R[c]	TTTGAGGCGAGGTGATAGGA
*Afpyr*G::*alcA*(p)::COG5 gene F	**ATCCTATCACCTCGCCTCAAA**ATGGCCTCC-GAACCCTC
*Afpyr*G::*alcA*(p)::COG5 gene R	AGAAGCTATCTCTCCTCCAGGC
*Afpyr*G::*alcA*(p)::COG5 upstream F	GTTTCTTTAATTCGCACCTCCG
*Afpyr*G::*alcA*(p)::COG5 upstream R	**TCAGTGCCTCCTCTCAGACAG**ACTGTCCCGATT-GTCCGC
*Afpyr*G::*alcA*(p)::COG5 fusion F	ACGACTACGTGGAAATCCATTG
*Afpyr*G::*alcA*(p)::COG5 fusion R	ACCCCTGTGAGACAGCGAC
*Afpyr*G::*alcA*(p)::COG5 confirmation F	TCCTCGGACTGGTGTTAAAAAG
*Afpyr*G::*alcA*(p)::COG5 confirmation R	GCGATGTTCATGAGTCCTGTC

[a]F = forward primer, R = reverse primer, *Afpyr*G = *Aspergillus fumigates pyr*G gene. [b]Bolded sequence indicates primer tails. Sequence "ATCCTATCACCTCGCCTCAAA" overlaps the *A. nidulans* AN11774 putative cog5 gene with the *alcA* promoter sequence, and the "TCAGTGCCTCCTCTCAGACAG" tail overlaps the promoter of the gene with the *Afpyr*G sequence of the *Afpyr*G::*alcA*(p) cassette. [C]Origin of primer is Gremillion et al. 2014.

ml in 5 ml of liquid, 1% glucose MM, and 1% glycerol MM in 35 × 10 mm Petri dishes at 28C for 17–24 h. Coverslips were then removed and placed onto clean glass microscope slides, viewed with an Olympus BX60 fluorescence microscope, and photographed using a Moticam ProS5 Plus digital camera and MoticImage Plus 3.0 software (Hong Kong, China). Photographs were then used to measure spore width (μm) using the MoticImage Plus 3.0 software. Spore width was measured once per spore. The widths of 200 total spores were measured with at least 29 spores measured per media type, per strain. A two-way analysis of variance (ANOVA) was used to determine the significance of the two independent variables: Media Type and Strain ($P \leq 0.05$). In the case of an interaction, individual statistical differences for each independent variable were determined with follow-up *t*-tests ($P \leq 0.05$).

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