Curr Genet (2017) 63:145–159
DOI 10.1007/s00294-016-0625-z

ORIGINAL ARTICLE

Regulation of *Neurospora crassa* cell wall remodeling via the *cot-1* pathway is mediated by *gul-1*

Inbal Herold[1] · **Oded Yarden**[1]

Received: 22 April 2016 / Revised: 14 June 2016 / Accepted: 16 June 2016 / Published online: 30 June 2016

Abstract Impairment of the *Neurospora crassa* Nuclear DBF2-related kinase-encoding gene *cot-1* results in pleiotropic effects, including abnormally thick hyphal cell walls and septa. An increase in the transcript abundance of genes encoding chitin and glucan synthases and the chitinase *gh18-5*, but not the cell wall integrity pathway transcription factor *rlm-1*, accompany the phenotypic changes observed. Deletion of *chs-5* or *chs-7* in a *cot-1* background results in a reduction of hyperbranching frequency characteristic of the *cot-1* parent. *gul-1* (a homologue of the yeast *SSD1* gene) encodes a translational regulator and has been shown to partially suppress *cot-1*. We demonstrate that the high expression levels of the cell wall remodeling genes analyzed is curbed, and reaches near wild type levels, when *gul-1* is inactivated. This is accompanied by morphological changes that include reduced cell wall thickness and restoration of normal chitin levels. We conclude that *gul-1* is a mediator of cell wall remodeling within the *cot-1* pathway.

Keywords *gul-1* · *Ssd1* · Cell wall remodeling · *cot-1* · Chitin synthase · *fks-1*

Communicated by M. Kupiec.

✉ Oded Yarden
Oded.Yarden@mail.huji.ac.il

[1] Department of Plant Pathology and Microbiology, The Robert H. Smith Faculty of Agriculture, Food and Environment, The Hebrew University of Jerusalem, 7610000 Rehovot, Israel

Introduction

Polar extension of hyphal cells, along with branching events, are characteristic hallmarks of the growth of filamentous fungi. Some of these processes in *Neurospora crassa* are regulated by COT-1 (colonial temperature sensitive-1) (Perkins et al. 1982; Yarden et al. 1992). This protein is a Nuclear DBF2-related (NDR) kinase, a member of the Ser/Thr kinase family that is involved in cell division and morphology spanning unicellular eukaryotes to mammals (Hergovich et al. 2006). Prominent changes in the temperature sensitive *cot-1* (ts) mutant include excessive branching as well as increased thickness of the cell wall and septa, which are evident when the strain is grown at restrictive temperatures of ≥32 °C (Yarden et al. 1992; Gorovits et al. 2000). Dysfunction of COT-1 homologues in other fungal species also leads to significant and pleiotropic morphological consequences (Durrenberger and Kronstad 1999; McNemar and Fonzi 2002; Buhr et al. 1996; Scheffer et al. 2005; Johns et al. 2006).

The fungal cell wall is essential for maintaining cell shape and integrity and its maintenance is a dynamic process that requires both deposition of new wall material and removal or rearrangement of existing linkages during growth and differentiation and in response to changing environmental conditions (Bowman and Free 2006; Osherov and Yarden 2010; Riquelme et al. 2011; Teparic and Mrsa 2013). Cell wall remodeling involves the function of biosynthetic enzymes (e.g., chitin and glucan synthases), which act in concert with enzymes exhibiting opposing activities (e.g., chitinases and glucanases). These enzymatic activities must be balanced to provide the cell wall elasticity and strength which allows new growth, branching, and morphological differentiation (Bowman and Free 2006). Based on their significance for fungal proliferation, cell wall remodeling

processes are prime potential targets for antifungal pharmaceuticals (Free 2013; Valiante et al. 2015).

The main constituent (30–80 % of cell wall mass) of the fungal cell wall is β-1-3-glucan (Free 2013). The glucan synthase complex (GSC) is comprised of a catalytic subunit (FKS1) and a regulatory subunit (ROH1). In contrast to yeasts, most filamentous fungi have only one FKS1 homolog (Tentler et al. 1997; Thompson et al. 1999; Beauvais and Latge 2001). In *N. crassa*, FKS-1 (NCU06871) was shown to be required for proper hyphal growth and development (Tentler et al. 1997). Impairing glucan synthase function has been shown to dramatically affect fungal cell morphology and growth and cell wall integrity (Free 2013).

Chitin content in most filamentous fungi has been reported to reach up to 15 % of the cell wall (Free 2013) and most of these fungi have seven or eight chitin synthase (*chs*) genes (Kong et al. 2012), even though up to ten clades of chitin synthase-encoding genes have been designated across different fungal linages (Li et al. 2016). All of the seven *chs* genes in *N. crassa* (Table 1) were found to be nonessential, suggesting redundancy in *chs* gene function (Beth Din and Yarden 1994; Beth Din et al. 1996; Dunlap et al. 2007; Riquelme and Bartnicki-Garcia 2008; Sanchez-Leon et al. 2011; Fajardo-Somera et al. 2015). Chitinases are also involved in a variety of functions associated with fungal growth (Kuranda and Robbins 1991; Dunkler et al. 2005; Baker et al. 2009). In *N. crassa*, 13 genes encoding for chitinases have been identified (Tzelepis et al. 2012).

The cell wall integrity (CWI) pathway is responsible for cell wall remodeling and reinforcement in response to cell wall stresses and is highly conserved among fungal species (Damveld et al. 2005; Fuchs and Mylonakis 2009). A key component of the CWI pathway in *Saccharomyces cerevisiae*, is the transcription factor Rlm1p (a MADS- box MEF2 type protein), which has been shown to be responsible for expression of numerous genes associated with the cell wall (Jung and Levin 1999). In various other fungi, Rlm1 has been shown to be involved in the regulation of expression of some cell wall-related biosynthetic enzymes (Damveld et al. 2005; Kovacs et al. 2013; Levin 2011; Delgado-Silva et al. 2014), yet the involvement of Rlm1p homologs in the CWI pathway in filamentous fungi is by far less clear than that established in *S. cerevisiae*.

Ssd1 is a *S. cerevisiae* mRNA-binding protein involved in translational regulation of cell wall remodeling proteins (Uesono et al. 1997; Hogan et al. 2008; Jansen et al. 2009; Kurischko et al. 2011; Wanless et al. 2014). This protein has been shown to associate with over 150 mRNA species, including those that encode proteins that function in cell wall organization and remodeling, cell separation and the "SUN" family (e.g., Sun4, Sim1, Uth1) of cell wall degrading proteins. Ssd1 can suppress translation of these cell proteins and this activity has been demonstrated to play an important role in cell integrity maintenance under stress conditions. Ssd1 function is negatively regulated by the yeast COT-1 homologue Cbk1 (Hogan et al. 2008; Jansen et al. 2009).

Similar to that found in yeasts, the *N. crassa* Ssd1 homologue, GUL-1, has also been shown to interact with the corresponding NDR kinase, COT-1. Suppressor analysis of *cot-1* (ts) showed that *gul-1* partially suppresses the pleiotropic morphology of the mutant (Terenzi and Reissig 1967). Morphological characteristics of *gul-1* mutants as well as the *gul-1* deletion strain include slow growth rate, reduced germination and hyperbranching (Terenzi and Reissig 1967; Seiler et al. 2006). Orthologues of Ssd1 have also been found in other filamentous fungi. In the plant pathogens *Colletotrichum lagenarium* and *Magnaporthe grisea* they were determined to be required for pathogenicity (Tanaka et al. 2007).

Table 1 *Neurospora crassa* strains used in this study

Strain	Genotype	Source	NCU number
Wild type	74-OR23-1 A	FGSC#987	
Wild type	ORS-SL6 a	FGSC#4200	
cot-1 (ts)	*cot-1* (C102t) A	FGSC#4065	NCU07296
cot-1 (ts)	*cot-1* (C102t) a	FGSC#4066	NCU07296
Δ *chs-1*	Δ *chs-1* A	FGSC#14319	NCU03611
Δ *chs-1*	Δ *chs-1* a	FGSC#14318	NCU03611
Δ *chs-2*	Δ *chs-2* A	FGSC#22804	NCU05239
Δ *chs-3*	Δ *chs-3* A	FGSC#14321	NCU04251
Δ *chs-3*	Δ *chs-3* a	FGSC#14320	NCU04251
Δ *chs-4*	Δ *chs-4* A	FGSC#18993	NCU09324
Δ *chs-4*	Δ *chs-4* a	FGSC#18992	NCU09324
Δ *chs-5*	Δ *chs-5* A	FGSC#11997	NCU04350
Δ *chs-5*	Δ *chs-5* a	FGSC#11996	NCU04350
Δ *chs-6*	Δ *chs-6* A	FGSC#13409	NCU05268
Δ *chs-6*	Δ *chs-6* a	FGSC#13408	NCU05268
Δ *chs-7*	Δ *chs-7* A	FGSC#21365	NCU04352
Δ *chs-7*	Δ *chs-7* a	FGSC#21364	NCU04352
Δ *chs-1*; *cot-1* (ts)	Δ *chs-1*; *cot-1* (ts)	Yarden lab	
Δ *chs-2*; *cot-1* (ts)	Δ *chs-2*; *cot-1* (ts)	This study	
Δ *chs-3*; *cot-1* (ts)	Δ *chs-3*; *cot-1* (ts)	Yarden lab	
Δ *chs-4*; *cot-1* (ts)	Δ *chs-4*; *cot-1* (ts)	Yarden lab	
Δ *chs-5*; *cot-1* (ts)	Δ *chs-5*; *cot-1* (ts)	Yarden lab	
Δ *chs-6*; *cot-1* (ts)	Δ *chs-6*; *cot-1* (ts)	Yarden lab	
Δ *chs-7*; *cot-1* (ts)	Δ *chs-7*; *cot-1* (ts)	Yarden lab	
Δ *rlm-1*	Δ *rlm-1* a	FGSC#14182	NCU02558
Δ *chit-1*	Δ *chit-1* a	FGSC#11678	NCU02184
Δ *gh18-5*	Δ *gh18-5* A	FGSC#12967	NCU04554
Δ *chit-1*; *cot-1* (ts)	Δ *chit-1*; *cot-1* (ts)	This study	
Δ *gh18-5*; *cot-1* (ts)	Δ *gh18-5*; *cot-1* (ts)	This study	
Δ*gul-1*	Δ *gul-1*	Seiler et al. (2006)	NCU01197
Δ *gul-1*; *cot-1* (ts)	Δ *gul-1*; *cot-1* (ts)	Seiler et al. (2006)	

Table 2 PCR primers used in this study

Name	Gene	Sequence	TM	Product size (bp)
chs1_2222For	*chs-1*	GTCATTTTGGTGGCCTTGGT	66	472
chs1_2694Rev	*chs-1*	CTTACCCCGATGCCAAATGT		
chs2_2292For	*chs-2*	TCTGGACAGCGACCTCAAGTTCAA	64	127
chs2_2419Rev	*chs-2*	TGCCAAAGGCGTTGAAGAACCATC		
chs3_992For	*chs-3*	GGCATGACCATCAAGAACGA	66.5	478
chs3_1470Rev	*chs-3*	TGTAGCTTCTCACCGGCAAA		
chs4_1718For	*chs-4*	GGGAGGTCGGTAGCAGTGTC	66	506
chs4_2224Rev	*chs-4*	TATCGGGGGTTGAGTGTTCC		
chs5_272For	*chs-5*	CCGTGCTGAGGATGGATGTA	66	507
chs5_780Rev	*chs-5*	TCGGCGGTAGGAAGATAGGA		
chs6_1684For	*chs-6*	GCTCTACCCCCTGATGTTCG	66	489
chs6_2173Rev	*chs-6*	TCGTTGGTGCTGCTGTCTTT		
chs7_5005For	*chs-7*	CCGTTCCCGTCTTCAGTTTC	66	500
chs7_5505Rev	*chs-7*	GGTCATCTCCAGCTCCTGCT		
chit-1_302For	*chit-1*	CTCGCGCTTTTCTCTGTCCA	64	426
chit-1_728For	*chit-1*	GCCTCGCAGCTAGGGTTGTT		
gh18-5_626For	*gh18-5*	GATTGGCGGGTGGACATACA	64	404
gh18-5_1030Rev	*gh18-5*	GGTCCATTCCTGGCAAGTCC		
gul-1_2403For	*gul-1*	GGTACTGCGGTCCATCTTGT	60	420
gul-1_2823Rev	*gul-1*	TTCAGTGGCCTGGCTAGACT		

Despite the aberrations in cell wall morphology that accompany impaired *cot-1* function, it has not yet been established to what extent *cot-1* is involved in the regulation of expression of genes encoding cell wall remodeling proteins. In this study, we report on the involvement of cell wall remodeling elements in conferring the *cot-1* (ts) phenotype. We propose that changes in the transcript abundance of genes encoding chitin and glucan synthases, as well as chitinases, contribute to the phenotypic changes observed. Furthermore, we show that these changes are partially mediated by *gul-1* but expression of the genes analyzed is not altered in a *N. crassa rlm-1* mutant.

Materials and methods

Strains, media and growth conditions

Standard procedures and media used in the handling of *N. crassa* were used as described by Davis (2000) or are available through the Fungal Genetics Stock Center (http://www.fgsc.net/Neurospora/NeurosporaProtocolGuide.htm). *N. crassa* strains used in this study are listed in Table 1. Strains were grown in either liquid or solid (supplemented with 1.5 % agar) Vogel's minimal medium with 1.5 % (w/v) sucrose (Vs). When required, the medium was supplemented with 10 μg ml^{-1} hygromycin B (Duchefa Biochemie, Haarlem, The Netherlands). In general, all mutants produced throughout this study were identified on the basis of morphological characteristics and their genetic nature was confirmed by PCR using specific primers (Table 2). For growth rate measurements, 6 μL of a conidial suspension (2×10^6 conidia/mL) were inoculated on Petri dishes containing Vs and growth was measured after 24 h.

qRT-PCR

Total RNA was isolated from mycelia grown for 16 h at 25 °C/28 °C/34 °C or grown for 12 h at 25 °C followed by 4 h at 34 °C, at 150 rpm, in liquid Vs. The RNA samples were purified with the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and DNA-free kit (Ambion). 1 μg of purified RNA was used for cDNA synthesis utilizing superscript II RNase H reverse transcriptase (Invitrogen, Carlsbad, California). Relative quantification of transcript abundance was performed on an ABI StepOnePlus Real-Time PCR sequence detection system and software (Applied Biosystems). For *chs* gene transcript detection, we used primers previously designed to identify unique 80–150 bp amplicons of the highly similar members of this gene family (Koch et al. 2014). PCR mixtures comprised a 10 μM concentration of each primer (Table 3), 5 μL of FAST SYBR green PCR master mix (Applied Biosystems), 2 μL of a 20 ng/μL cDNA solution and nuclease-free water to

Table 3 PCR primers used for transcriptional analysis

Name	Gene	Sequence	TM	Product size (bp)
BT_92For	*tub-2*	ATGCCTCCGGTGTGTACAATG	64	79
BT_171Rev	*tub-2*	GTTGCCGGAAGCCTCGTT		
chs1_2389For	*chs-1*	GTGGGGTACCAAGGGTTCGG	64	104
chs1_2493Rev	*chs-1*	CCTGTGGCTTCTCAATCTCT		
chs2_2292For	*chs-2*	TCTGGACAGCGACCTCAAGTTCAA	64	127
chs2_2419Rev	*chs-2*	TGCCAAAGGCGTTGAAGAACCATC		
chs3_1003For	*chs-3*	TCAAGAACGATGTCGTCCAGCTCA	64	116
chs3_1119Rev	*chs-3*	CAAAGGCCTGGAAGAACCAACGAT		
chs4_2120For	*chs-4*	TCTGGACTCGATTGCAATGACGGA	64	90
chs4_2210Rev	*chs-4*	TTCCTCTCCGTGGCCCTTGATAAT		
chs5_471For	*chs-5*	ACCCTCAACCTTACAGGCAACCTT	64	81
chs5_552Rev	*chs-5*	CAACAAGCCTTTGTCGGTGTCGAT		
chs6_1844For	*chs-6*	AAGACGGGTGACGACCTCAA	64	99
chs6_1943Rev	*chs-6*	TAATGCCGGTGGTGAAGCCC		
chs7_5164For	*chs-7*	AAATCTCGGGCTTCTCATACGCCA	64	146
chs7_5310Rev	*chs-7*	AACGGGCAGATGTGTCTTATCGCT		
fks-1_1559For	*fks-1*	ACTCCCATGTACGAACAGCAGGTT	64	106
fks-1_1665Rev	*fks-1*	GTTGCTCCAAAGTTGATCAGCGCA		
rlm-1_38For	*rlm-1*	TGACAGGAATCGCTCTGTAACATT	64	131
rlm-1_169Rev	*rlm-1*	ATTCGTACAGCTTTTTGTTGTTGC		
chit-1_940For	*chit-1*	AGACTTTGGCCAATGGAATGACTT	64	86
chit-1_1026Rev	*chit-1*	GAACTCAGAGCTCAGGTCAACAGC		
gh18-5_168For	*gh18-5*	GCAACGACAGCTACAGTCACCTTT	64	117
gh18-5_285Rev	*gh18-5*	ACTTGTTTCTGTTGTGGCTGGTGT		

a final volume of 10 μL. The annealing temperature was 64 °C. Total cDNA abundance in the samples was normalized using the beta tubulin (*tub-2*) gene (Table 3). In all experiments, samples were amplified in triplicate, and the average cycle threshold was then calculated and used to determine the relative expression of each gene. Relative expression levels were calculated using the $2^{-\Delta Ct}$ method.

Microscopy

Light and fluorescent microscopy was performed with an EVOS FL auto imaging system (Life Technologies). Strains were grown on slides coated with solid (supplemented with 1.5 % agarose) Vs medium at 25 °C/28 °C for 16 h or grown for 12 h at 25 °C followed by 4 h at 34 °C. To determine general chitin deposition in cell wall and septa the strains were stained with calcofluor white (10 mg/mL). To determine the length between branches we used the ImageJ 1.37 V software package (Rasband, WS, US National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij/, 1997–2006).

For transmittance electron microscopy (TEM), strains were grown on solid Vogel's minimal medium. Samples were prepared on the basis of a previously described protocol (Gorovits et al. 2000) with slight modifications. Hyphal samples (0.5 × 0.5 cm squares) were cut from the agar and fixed in 1.5 % $KMnO_4$ (w/v in H_2O) for 20 min at room temperature. The samples were rinsed three times in double distilled water (DDW), and three times for 10 min each, in cacodylate buffer and then washed once in DDW. The samples were then washed in DDW and were subjected to en bloc staining with 1 % uranyl acetate in DDW (overnight, at room temperature) to enhance contrast. Samples were washed four times for 10 min in 50 % ethanol, followed by dehydration in increasing concentrations of ethanol consisting of 70, 80, 90, 95 %, (10 min each) followed by 100 % anhydrous ethanol three times (20 min), and twice in propylene oxide (10 min). Following dehydration, the tissues were infiltrated with increasing concentrations of agar 100 resins in propylene oxide, consisting of 25, 50, 75, and 100 % resin (16 h for each step). The tissues were then embedded in fresh resin and polymerized in an oven at 60 °C for 48 h. Embedded sample blocks were sectioned with a diamond knife on an LKB 3 microtome and ultrathin Sects. (80 nm) were collected onto 200 Mesh, thin bar copper grids. The gridded sections were sequentially stained with uranyl acetate and lead citrate for 10 min each and viewed with a Tecnai 12 TEM 100 kV (Phillips,

Eindhoven, The Netherlands) microscope equipped with a megaview II CCD camera and Analysis® version 3.0 software (SoftImaging System GmbH, Münster, Germany).

Cell wall carbohydrate analyses

Cell wall samples were prepared as described by Maddi and Free (2010). Briefly, cell walls were isolated from cells grown for 12 h on Vs medium at 25 °C followed by 4 h at 34 °C, at 150 rpm. Carbohydrate analyses were performed at the University of Georgia Complex Carbohydrate Research Center (Athens, GA). For glycosyl linkage analysis, the samples were permethylated, depolymerized, reduced, and acetylated, and the partially methylated alditol acetates were analyzed by gas chromatography-mass spectrometry (GC–MS), essentially as described by Heiss et al. (2009), with minor modifications to accommodate the detection of amino sugars. Approximately 1 mg of cell wall sample was used for linkage analysis. The sample was suspended in 200 µl of dimethyl sulfoxide and left to stir for 3 days. Permethylation was affected by two rounds of treatment with sodium hydroxide (15 min) and methyl iodide (45 min). Following sample workup, the permethylated material was hydrolyzed using 2 M trifluoroacetic acid (2 h in sealed tube at 121 °C), reduced with $NaBD_4$, and acetylated using acetic anhydride/pyridine. The resulting partially methylated alditol acetates were analyzed on an Agilent 7890A GC interfaced to a 5975C MSD (mass selective detector, electron impact ionization mode); separation was performed on a 30 m Supelco SP-2331 bonded phase fused silica capillary column.

Results

Transcript levels of *chs* family members and *fks-1* are increased in a *cot-1* (ts) background

To analyze the possible association between *cot-1* and genes involved in cell wall biosynthesis, we first examined the transcript levels of chitin and glucan synthases (*chs-1–7* and *fks-1,* respectively) in a *cot-1* (ts) background. The transcript abundance of the *chs* genes and *fks-1* was assayed in RNA isolated from hyphae of wild type and *cot-1* (ts) strains grown at 25 °C, as well as following a shift to the *cot-1* (ts) restrictive temperature (34 °C). On the basis of the differential expression pattern of *chs-1–7* in the wild type control, we determined that the *N. crassa chs* genes can be divided into two major groups, based on their transcript abundance: Highly expressed (*chs-1, 3, 4, 5, 7*) and those expressed at lower levels (*chs-2, 6*). When *cot-1* (ts) was grown at 25 °C, no distinct differences were observed between the expression patterns of the *chs* and *fks-1* genes, when compared to the wild type (Fig. 1). However, at restrictive temperature, a statistically significant increase in the expression of the highly expressed group of *chs* genes (*chs-1, 2, 3, 4*), as well of *fks-1*, was observed in a *cot-1* (ts) background. The most pronounced change was observed in expression of *chs-1*, where transcript abundance was almost threefold of that measured in the control. Overall, the increases observed in the expression of the cell wall biosynthetic enzyme-encoding genes indicate that they are involved in some of the morphological cell wall defects characteristic of *cot-1* (ts).

Deletion of *chs-5* or *chs-7* result in a reduction in *cot-1* (ts) hyperbranching

The fact that deletion/inactivation of some (but not all) single *chs*-encoding genes in *N. crassa* results in various changes in hyphal growth rates has been previously described (Beth Din and Yarden, 1994; Beth Din et al. 1996; Sanchez-Leon et al. 2011; Fajardo-Somera et al. 2015). To verify the differences in growth rates of the different mutants under the conditions performed in this study, we first carried out comparative growth tests in which all *chs* deletion strains were examined in parallel. Based on our results, we determined that Δ*chs-2* and Δ*chs-4* grew in a manner similar to the wild type. In contrast, Δ*chs-1,* Δ*chs-3* and Δ*chs-7* exhibited slower growth (80 % of wild type), while Δ*chs-5* and Δ*chs-6* grew at only 35 % of the rate measured in the wild type. Excluding the case of the slow growing Δ*chs-5*, results obtained were in line with those recently described (Fajardo-Somera et al. 2015).

To determine the possible genetic interactions between the genes encoding CHSs and COT-1, we produced seven *chs*;*cot-1* (ts) double mutants. To follow the possible effects of *chs* gene deletion in a *cot-1* (ts) background, we cultured the double mutants at 28 °C, a semi-restrictive temperature, which allows measurable growth of *cot-1* (ts) and detecting even subtle morphological changes. Radial growth of most of the double mutants was comparable to that of *cot-1* (ts). The exceptions were Δ*chs-1;cot-1* (ts), Δ*chs-5;cot-1* (ts) and Δ*chs-6;cot-1* (ts), which grew at slower rates, probably due to the additive effects of the mutations in *cot-1* (ts) and the slower growing Δ*chs-1,* Δ*chs-5* and Δ*chs-6* strains. Nonetheless, even though growth rates were different, no visible differences in colony morphology or in hyphal structures was observed between the double mutants and *cot-1* (ts).

When cultures were shifted from 28 to 34 °C, all double mutants exhibited a morphology similar to *cot-1*, excluding Δ*chs-5;cot-1* (ts) and Δ*chs-7;cot-1* (ts). These exhibited a less severe colonial phenotype, which was characterized by a reduction in hyphal density and reduced branching frequencies of the colony (Fig. 2). Thus, while the distance between

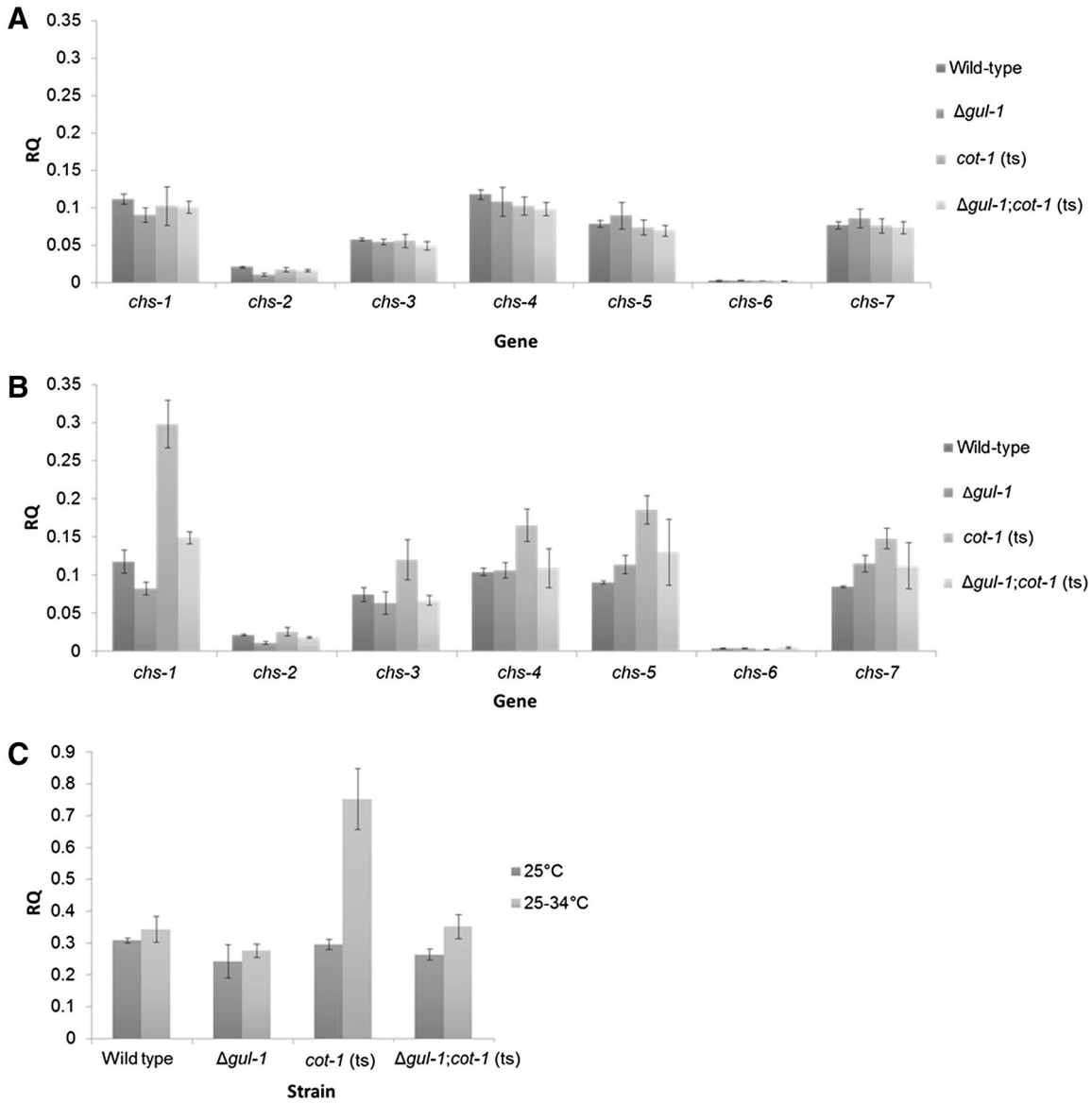

Fig. 1 Expression profiles of *chs* genes (**a**, **b**) and *fks-1* (**c**) in wild type, *cot-1* (ts) Δ*gul-1* and Δ*gul-1*;*cot-1* (ts) mutants grown at permissive temperature (a) or following a shift to *cot-1* (ts) restrictive temperature (b), and *fks-1* (**c**) in the different mutants as determined by qRT-PCR. RNA was purified from conidia germinated in liquid Vs medium for 16 h at 25 °C or for 12 h at 25 °C followed by 4 h at 34 °C. The relative expression level of individual genes was analyzed by the $2^{-\Delta Ct}$ method with the β-tubulin gene as the internal control for normalization. Bars indicate ±1.96SE (confidence level of 95 %), determined with data obtained from three independent replicates

branches in the Δ*chs-5* and Δ*chs-7* strains was comparable to the wild type (~160 µM), and was only 10 µM in *cot-1* (ts), the distance in the double mutants ranged 30–40 µM. Swelling of some of the hyphal cells was also observed. In all strains, calcofluor white staining patterns were similar, suggesting no drastic changes in chitin deposition had occurred.

Based on these results, we concluded that at least two chitin synthases (*chs-5* and *chs-7*) have a genetic interaction with *cot-1* (ts), as determined on the basis of the colony morphology observed.

Two chitinases (*chit-1*, *gh18-5*) are expressed in opposing manners in *cot-1* (ts)

chit-1 and *gh18-5* transcripts were analyzed due to the suggested involvement of the corresponding proteins in the CWI pathway (Seidl 2008; Kovacs et al. 2013; Yamazaki et al.

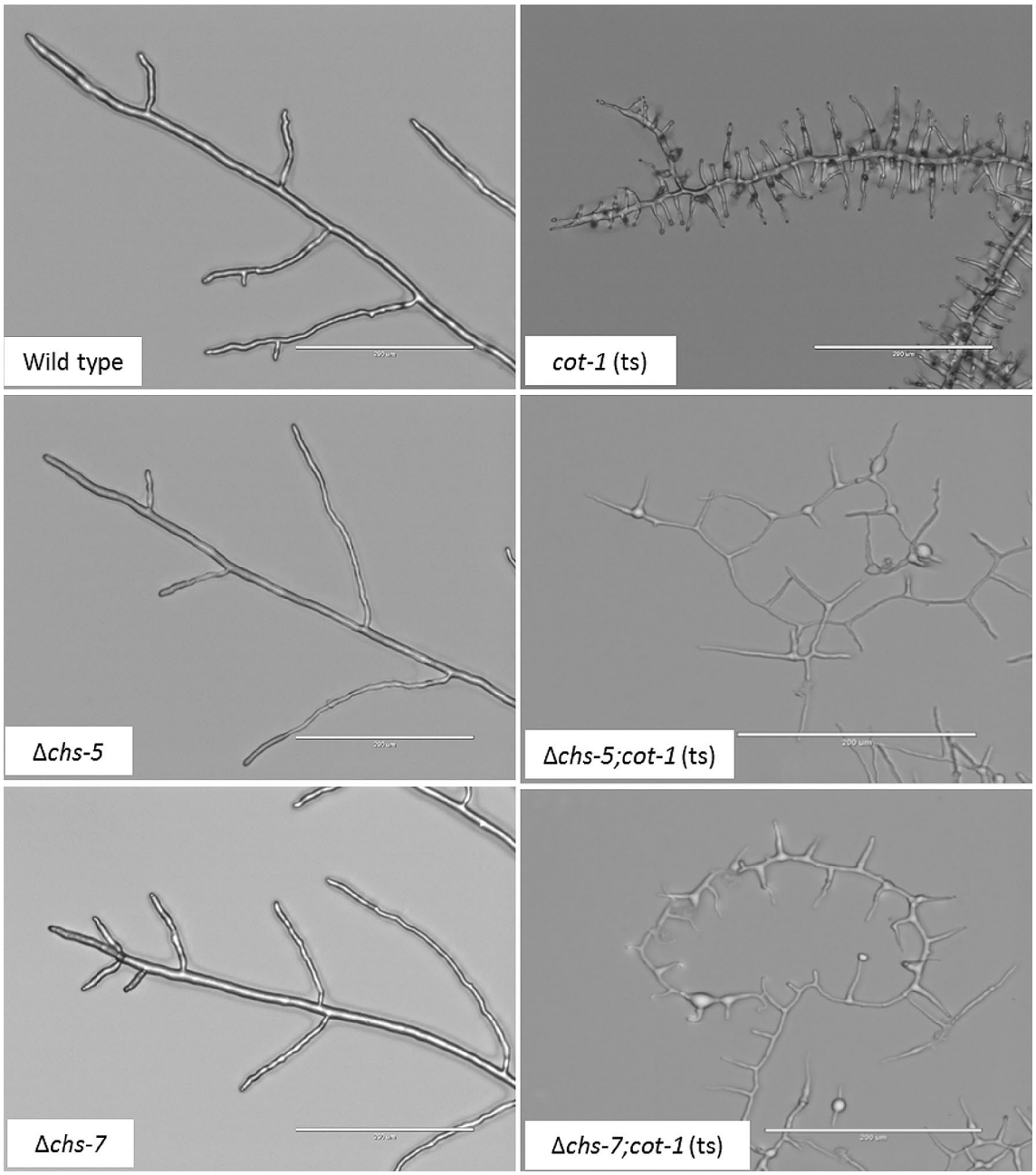

Fig. 2 Deletion of *chs-5* or *chs-7* results in partial suppression of the *cot-1* (ts) phenotype. Cultures were grown on slides coated with Vs medium for 12 h at 28 °C followed by 4 h at 34 °C. Bar 200 µm

2008). When grown at 25 °C, RNA levels of both *chit-1* and *gh18-5* in the *cot-1* (ts) mutant and in the wild type strain were comparable (Fig. 3). However, when the wild type was subjected to a 25–34 °C temperature shift, *chit-1* expression decreased significantly (by ~60 %). A more marked decrease was observed in *cot-1* (ts), where *chit-1* expression was less than 20 % of that measured prior to the shift. The expression pattern of *gh18-5* in both the wild type and *cot-1* (ts) was different than that of *chit-1*. Transcript levels were not altered in the wild type following the temperature shift. However, in the *cot-1* (ts) mutant, following the temperature shift, *gh18-5* transcript abundance was nearly tenfold higher.

These results show that the two chitinase-encoding genes exhibit opposing expression patterns in a *cot-1* (ts) background, and that *cot-1* inactivation emphasizes the different roles these genes may play in cell wall remodeling.

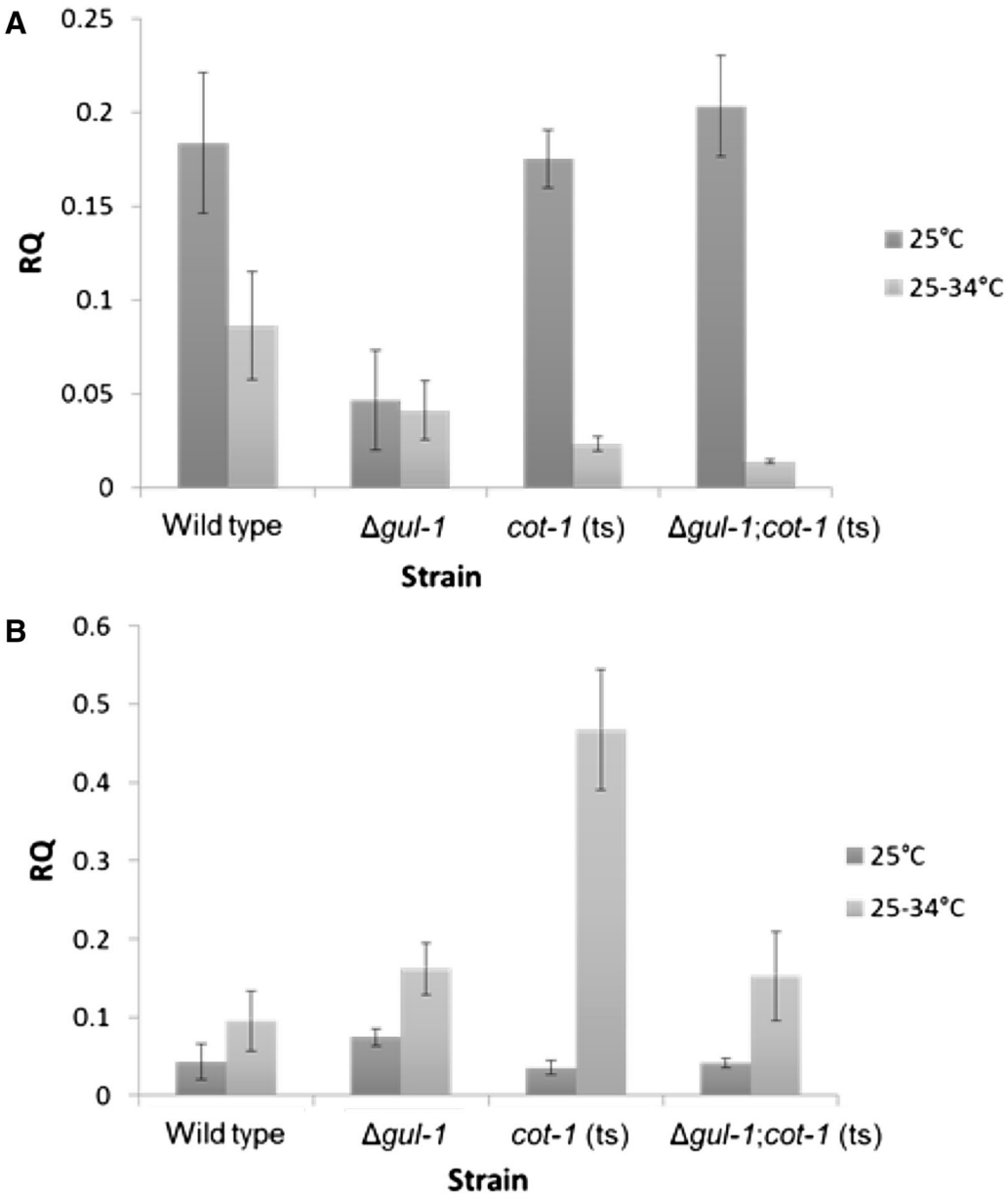

Fig. 3 Expression profiles of *chit-1* (**a**) and *gh18-5* (**b**) in wild type, Δ*gul-1*, *cot-1* (ts) and Δ*gul-1*;*cot-1* (ts) strains as determined by qRT-PCR. RNA was purified from conidia germinated in liquid Vs medium for 16 h at 25 °C or for 12 h at 25 °C followed by 4 h at 34 °C. The relative expression level of individual gene was analyzed by the $2^{-\Delta Ct}$ method with the β-tubulin gene as the internal control for normalization. Bars indicate ±1.96SE (confidence level of 95 %), determined with data from three independent replicates

Deletion of *chit-1* in a *cot-1* (ts) background results in a significant reduction in growth rate

As chitinases are components of the cell wall maintenance and remodeling machinery, we first examined the consequences of *chit-1* and *gh18-5* deletion. Interestingly, we found that the linear growth rates of Δ*chit-1* and Δ*gh18-5* did not differ from that of the wild type strain. To determine whether *chit-1* and *gh18-5* genetically interact with *cot-1* (ts), double mutants of the chitinases and *cot-1* (ts) were produced. Δ*chit-1*;*cot-1* (ts) exhibited a significant reduction in growth rate when compared to the parental strains at the different growth conditions [80 and 70 % of Δ*chit-1* and *cot-1* (ts), respectively]. However, the morphology of the mutant was similar to *cot-1* (ts). In contrast to *Δchit-1*;*cot-1* (ts), Δ*gh18-5*;*cot-1* (ts) exhibited a similar growth rate to the parental strains at the different growth conditions [e.g., similar to Δ*gh18-5* at 25 °C and to *cot-1* (ts) at 34 °C].

These results demonstrate that under the conditions tested, *chit-1*, but not *gh18-5*, genetically interact with *cot-1*.

Inactivation of *cot-1* does not confer changes in *rlm-1* expression

The *A. nidulans* Rlm1A protein has been shown to be involved in stress-related CWI pathway regulation (Kovacs et al. 2013). The most likely homologue (51 % amino acid sequence identity) of this gene in *N. crassa* (NCU02558) has not yet been functionally analyzed. To investigate the possible involvement of *rlm-1* in changes in cell wall remodeling in *cot-1* (ts), we analyzed this mutant and studied the expression of the relevant genes in the Δ*rlm-1* strain.

The *N. crassa* Δ*rlm-1* strain grew at a rate that was only 5 % slower than the wild type, suggesting that the requirement for a functional *rlm-1* is marginal for proper growth and development of *N. crassa* under standard conditions.

As *cot-1* has been shown to exhibit characteristics of stress-related conditions (Gorovits and Yarden 2003), we examined *rlm-1* expression in *cot-1* (ts). The expression level of *rlm-1* was only slightly increased following a 25–34 °C shift in *cot-1* (ts), when compared to the wild type.

We further examined the possible relevance of *rlm-1* for cell wall remodeling and measured the transcript abundance of *chs*, *fks-1* and chitinase genes in the Δ*rlm-1* mutant. In all cases, the expression levels of *chs-1–7* and *fks-1* in the mutant were similar to those measured in the wild type strain. In addition, no changes in the expression of *chit-1* and *gh18-5* were observed in the mutant. We concluded that the cellular stresses imposed by impairment of COT-1 do not activate the CWI pathway via *rlm-1*.

A mutation in *gul-1* reduces the severity of the *cot-1* (ts) phenotype and is accompanied by altered cell wall remodeling gene expression

Previous studies have shown that the growth defects of *cot-1* (ts) are partially suppressed in a *gul-1* background, suggesting a functional link between GUL-1 and COT-1 (Terenzi and Reissig 1967; Seiler et al. 2006). To determine the possible role of *gul-1* as a mediator between *cot-1* (ts) and genes involved in cell wall remodeling, we examined the transcript levels of *chs-1–7* and *fks-1* in a Δ*gul-1;cot-1* (ts) mutant. At 25 °C, the expression levels of the cell wall biosynthetic genes were similar to those measured in the parental strains and the wild type (Fig. 1). Following a 25–34 °C temperature shift, *chs-1, 2, 3, 4,* as well as *fks-1* expression, significantly increased in the *cot-1* (ts) mutant, as described above (Fig. 1). However, their expression levels in the Δ*gul-1;cot-1* (ts) and the parental strains were similar to the wild type (Fig. 1). These results show that the increases in cell wall biosynthetic gene expression patterns in a *cot-1* (ts) background are dependent on a functional *gul-1*.

We also examined the transcript levels of *chit-1* and *gh18-5* in the *Δgul-1* and *Δgul-1;cot-1* (ts) mutants. When Δ*gul-1;cot-1* (ts) was grown at 25 °C, no distinct differences were observed between the expression patterns of the *chit-1* gene when compared to *cot-1* (ts) and the wild type (Fig. 3). However, the expression of *chit-1* in a Δ*gul-1* mutant was only about 30 % of that measured in the wild type. When a 25–34 °C temperature shift was imposed, *chit-1* expression decreased significantly in the wild type, yet this gene's expression was not altered in Δ*gul-1*. Deletion of *gul-1* in a *cot-1* background did not alter the already reduced expression of *chit-1* previously measured in the *cot-1* (ts) parent (at 34 °C). Thus, the decrease in the expression of *chit-1* in a *cot-1* (ts) background is independent of the presence of *gul-1*.

The expression level of *gh18-5* in Δ*gul-1;cot-1* (ts) was similar to the parental strains and the wild type at 25 °C. However, in contrast to that observed in *cot-1* (ts), following a shift to restrictive temperature, the change in transcript abundance of *gh18-5* in the Δ*gul-1;cot-1* (ts) strain was negligible, when compared to the several fold increase observed in the *cot-1* (ts) parent (Fig. 3). Taken together, our results suggest that GUL-1 is most likely a functional link between COT-1 and the *N. crassa* cell wall remodeling proteins CHS-1, 2, 3, 4, as well as FKS-1 and GH18-5.

A mutation in *gul-1* suppresses the effect of impaired COT-1 function on the cell wall

To further investigate the contributions of *gul-1* in diminishing the effect of the *cot-1* (ts) mutation on the cell wall, hyphae were examined using transmission electron microscopy. Hyphal cell walls of the *cot-1* (ts) strain were thicker and the septa showed a multilayered structure when compared with the wild type [as was previously described by (Gorovits et al. 2000)]. In the Δ*gul-1* mutant, the cell wall appeared to be thinner than the wild type, whereas the Δ*gul-1*;*cot-1* (ts) double mutant exhibited septa and cell wall morphology which highly resemble that of the wild type (Fig. 4). These observations were supported, at least in part, by carbohydrate cell wall composition analysis (Table 4). The major change observed was that of chitin content, which was increased by almost 40 % in the *cot-1* (ts) strain when compared to the wild type. Chitin content was reduced by 10 % in the Δ*gul-1* strain, but close to wild type levels in the Δ*gul*-1;*cot-1* (ts) double mutant. Interestingly, the changes in glucan (the major cell wall carbohydrate constituent) were much lower, while at the same time the deletion of *gul-1* resulted in a 20 % reduction in

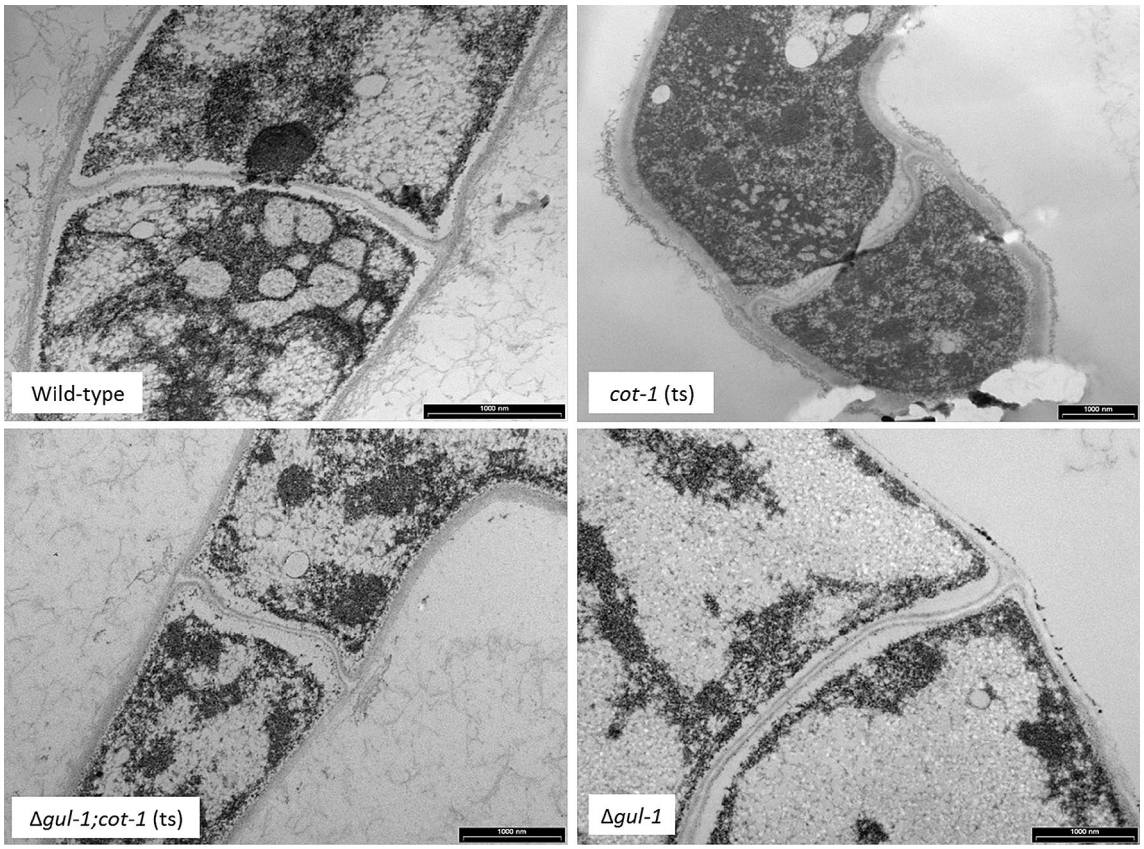

Fig. 4 Transmission electron micrographs of hyphal morphology of wild type, *cot-1*, Δ*gul-1* and Δ*gul-1*;*cot-1* strains. Cultures were grown on Vs medium for 12 h at 25 °C followed by 4 h at 34 °C. Bar 1000 nm

Table 4 Carbohydrate content in the *cot-1* (ts), Δ*gul-1* and Δ*gul-1*;*cot-1* (ts) mutants (percent, relative to wild type)

Glycosyl residue	*cot-1* (ts)	Δ*gul-1*	Δ*gul-1*;*cot-1* (ts)
Glucan	92.9	113.4	109.0
Mannan	99.4	81.0	76.6
Chitin	139.5	87.1	107.2

mannan. The Δ*gul-1* mutant was also found to be more sensitive to a chitin synthase inhibitor (growth rate of ~40 % of the wild type in the presence of 10^{-5} M Nikkomycin Z). Based on these results, we suggest that GUL-1 contributes to changes in cell wall morphology and composition in a manner that confers the suppressive effect on *cot-1*.

Discussion

In this study, we examined the relationship between a key component controlling the polar development of hyphae in the fungus *N. crassa* and the downstream elements that contribute to determination of cell wall structure.

The Ndr kinase COT-1 is important for cell differentiation and polar morphogenesis in various fungi. One of the outcomes of impairing *cot-1* function is the presence of thick hyphal cell walls and septa (Gorovits et al. 1999, 2000; Yarden et al. 1992). This implies the occurrence of changes in the function of the cell wall biosynthetic machinery, predominantly chitin and glucan synthases. Glucan is the major constituent of the fungal cell wall (Maddi et al. 2009; Free 2013). However, even though chitin comprises only a small amount of the fungal cell wall, subtle changes in its concentration can have a large impact on fungal morphology (Hall 2015).

To analyze the possible association between *cot-1* (ts) and genes involved in cell wall biosynthesis, we first studied the expression level of the chitin synthases gene family (*chs-1–7*) in *N. crassa*. On the basis of the differential expression pattern of these genes, we determined that *chs* genes can be divided into two major groups: highly expressed: *chs-1, 3, 4, 5, 7* (class III, I, IV, V, VII, respectively) and those expressed at lower levels: *chs-2, 6* (class II and class VI, respectively). Even though this pattern was repeatedly observed, it is still unclear if this division has functional relevance with regard to cell wall synthesis. In

M. oryzae, CHS1-CHS7 designation was based on their orthologs in *N. crassa.* Nevertheless, the expression patterns of these genes are not identical in both fungi (Kong et al. 2012). Hence, it is likely that similar class designation does not necessarily imply identical expression/function.

Examining the transcription level of chitin synthases and glucan synthase genes showed that there is an increase of 20–250 % in the expression of these genes in a *cot-1* (ts) background. These results show that changes of those elements may contribute to the phenotype of *cot-1* with regard to the abnormally thick cell wall and septa. To what extent the variation in changes of expression of the different *chs* genes is due to their functional redundancies, has yet to be determined. This is further emphasized by the analysis of the *chs*;*cot-1* (ts) double mutants (see below).

In *S. cerevisiae*, deletion of *CBK1* alters the pattern of transcription of genes involved in cell wall morphology and those that are involved in digestion of the chitinous septum between mother and daughter cells. Our results were similar to those described by Bidlingmaier et al. (2001), who showed that a chitin synthase encoded by *CHS1* was significantly elevated in *cbk1*D cells. Thus, our results along with evidence obtained in *S. cerevisiae* and *C. albicans* (McNemar and Fonzi 2002) suggest that it is possible that the polarized growth defects observed in *cot-1* (ts) are due, at least in part, by misregulation of the expression of genes important for cell wall remodeling-related processes.

Characterization of seven chitin synthases deletion mutants in a *cot-1* (ts) background revealed that deletion of single *chs* genes did not result in improved radial growth of *cot-1* (ts), probably due to the redundancy of gene family members. *chs* gene redundancy has been clearly demonstrated in *A. nidulans,* where *chsA* (class II) and *chsD* (class IV) have highly similar functions in conidia formation (Motoyama et al. 1996). Two additional chitin synthases, *CsmA* (class V) and *CsmB* (class VII), carry out overlapping functions that are required for hyphal tip growth (Takeshita et al. 2006). Several *chs* gene double mutants in *N. crassa* also do not exhibit severe phenotypes, further supporting the partially redundant nature of some of the *chs* family members (Herold and Yarden, unpublished).

Microscopic analysis of Δ*chs-5;cot-1* (ts) and Δ*chs-7;cot-1* (ts) hyphae showed that these mutants exhibited a reduction in branching when compared to *cot-1* (ts), an indication for the presence of genetic and functional interactions between them. The *N. crassa* genes encoding for CHS5 and CHS7 belong to class V and VII chitin synthases, respectively. Both of these classes have a conserved myosin motor-like domain (MMD) at their amino termini (Fujiwara et al. 1997; Riquelme and Bartnicki-Garcia 2008). In *A. nidulans, CsmA* (class V) has been localized near actin structures at the hyphal tips and at forming septa, and it was shown that the MMD was, in fact, capable of binding to actin (Takeshita et al. 2006). In *A. fumigatus*, deletion of the *CSMA* and *CSMB* genes (both harboring an MMD) induced significant disorganization of the cell wall structure, even though no modifications of chitin content in their conidial cell walls were reported (Jimenez-Ortigosa et al. 2012). Based on changes in actin patch deposition patterns in the *cot-1* (ts) and *cot-1* (T589A) strains, Ziv et al. (2009) suggested a functional link between COT1 and some of the components regulating the actin cytoskeleton and factors involved in establishing and/or maintaining cell wall integrity. The fact that the two *chs* genes whose deletion conferred a phenotypic change in *cot-1* (ts) encode a protein that harbors an MMD, supports this suggestion.

To address the question whether the *cot-1* (ts) phenotype may also be an outcome, at least in part, of impaired regulation of cell wall degrading enzymes, we analyzed the changes in expression of two chitinase encoding genes—*gh18-5* and *chit-1*. The *N. crassa* GH18-5 belongs to a highly conserved chitinase A group, whose orthologs are present in many ascomycetes (Karlsson and Stenlid 2008). The *N. crassa chit-1* gene belongs to the B1 group and its expression was found to be induced by colloidal chitin (Tzelepis et al. 2012). Several reports have suggested that subgroup B chitinases could be responsible for cell wall remodeling in fungi (Adams 2004; Jaques et al. 2003; Hurtado-Guerrero and van Aalten 2007). *chit-1* influences growth rate of *N. crassa*, presumably through a cell wall plasticizing role (Tzelepis et al. 2012). The *A. nidulans* ortholog ChiA is a GPI-anchored chitinase that is localized at hyphal branching sites and at hyphal tips in conidial germ tubes (Yamazaki et al. 2008). In this study, we found that the expression of both *chit-1* and *gh18*-5 was altered in a *cot-1* (ts) background. These changes could well affect hyphal extension and branching in a manner that contributes to the *cot-1* (ts) phenotype. These results are comparable to the reported in *S. cerevisiae*, where the cell separation defects in the Δ*cbk1* strain were preceded by a drastic reduction in the expression of *CTS1* (a *chit-1* homologue) (Racki et al. 2000; Bidlingmaier et al. 2001). In *Pneumocystis carinii, CBK1* expression stimulated transcription of the *CTS1* chitinase which is necessary for cell wall separation in that fungus (Kottom and Limper 2004).

Both Δ*chit-1* and Δ*gh18-5* strains were morphologically similar to the wild type. These results are partially in line with a previous study (Tzelepis et al. 2012), where Δ*gh18-5* was shown to be similar to the wild type. However, under the growth conditions described in that report, Δ*chit-1* was shown to exhibit several phenotypic defects. The severe phenotype observed in the Δ*chit-1;cot-1* (ts) strain established the presence of a genetic interaction between the two genes. On the basis of the expression data we further concluded that a functional *cot-1* (ts) is required

for proper *chit-1* expression and that impaired expression of *chit-1* confers developmental defects. No genetic interaction was observed between *gh18-5* and *cot-1* (ts) and the additive effects observed indicate that these elements probably operate in different pathways.

Mechanistic analysis of NDR protein function and identifying proteins that interact with them has been studied in several systems (Hanks and Hunter 1995; Hergovich et al. 2006; Pearce et al. 2010). However, information regarding downstream elements affected by the NDR complex is limited. In this study, we have shown, for the first time, the involvement of cell wall remodeling elements in the *cot-1* (ts) phenotype. We propose that this may well be due to changes in the transcription of some genes encoding chitin and glucan synthases and chitinases we have observed.

The fact that *cot-1* (ts) undergoes phenotypic suppression when a wide variety of stresses are imposed indicates that when *cot-1* function is impaired, cellular stress responses are activated. One explanation for the environmental suppression that occurs under such circumstances is that the balance between the external stresses and the preactivated stress response in the *cot-1* mutant enables the development and hyphal extension of the fungus (Gorovits and Yarden 2003). Some of these stresses are known to activate the CWI pathway.

Is *rlm-1* involved in mediating CWI element expression in *N. crassa*? In *S. cerevisiae* and *C. albicans*, RLM1 has been shown to play key roles in CWI (Dodou and Treisman 1997; Watanabe et al. 1997; Delgado-Silva et al. 2014). In filamentous fungi, information concerning the involvement of *rlm-1* in cell wall remodeling is less clear and in some cases even contradicting (Damveld et al. 2005; Fujioka et al. 2007, Kovacs et al. 2013). We have found that inactivating *rlm-1* results in only negligible effects on growth and development of *N. crassa.* Analyzing the expression of *rlm-1* in the *cot-1* (ts) mutant demonstrated that *rlm-1* is most likely not involved in conferring the *cot-1* phenotype. Furthermore, deletion of *rlm-1* did not result in changes in *chs*, *fks-1* as well as *gh18-5* and *chit-1* expression. This is similar to that found in *A. nidulans*, where most cell wall-related biosynthetic genes were regulated independently of RlmA, with the exception of 1,3-α glucan synthase (Fujioka et al. 2007). However, Kovacs et al. (2013) reported that expression of some of the *A. nidulans* cell wall hydrolase-encoding genes is, in fact, regulated by RlmA. Overall, results obtained here demonstrate that in spite of the structural conservation of RLM-1, its function in *N. crassa* is different from that observed in several other fungi.

We also analyzed the significance of *gul-1* in conferring the pleiotropic phenotype of *cot-1* (ts). We found that the expression of several cell wall biosynthetic enzyme-encoding genes (*chs-1*, *chs-2*, *chs-3*, *chs-4* and *fks-1*) was found to be regulated in a *gul-1* dependent manner. When expression levels of *chs-1–7* and *fks-1* were examined in a *gul-1*;*cot-1* (ts) background, the increase in their expression, as was determined to occur in *cot-1* (ts), was significantly curbed. These results further support the hypothesis that miss-expression of the cell wall remodeling machinery contributes to the severity of the *cot-1* (ts) phenotype and suggest that *gul-1* is a functional link between *cot-1* and the cell wall biosynthetic elements in *N. crassa*.

In yeast, loss of Cbk1 regulation of Ssd1 causes lethal defects in cell wall organization, likely by making the cell wall too rigid to expand with the growing bud (Jansen et al. 2009). To determine whether *gul-1* is also a mediator between *cot-1* and cell wall degrading elements in a filamentous fungus, we quantified the mRNA levels of *gh18-5* and *chit-1* in the relevant backgrounds. The increase in the expression of *gh18-5* was found to be dependent on a functional *gul-1*. The decrease in the expression of *chit-1* (in a *cot-1* background) was found to be independent of the presence of *gul-1*. Thus, *gul-1* is probably not a functional link between *cot-1* and *chit-1* and *gul-1* and *cot-1* may affect *chit-1* in different, independent, pathways.

Taken together, our results indicate that the presence of *gul-1* is required for the increase in the mRNA levels of cell wall remodeling genes in a *cot-1* (ts) background. This increase can partially contribute to the morphological defects of *cot-1* (ts), and in particular, to the abnormal thick cell wall and septa. Similar results suggest that one of the functions of Cbk1 in hyphal morphogenesis of *C. albicans* is down-regulation of the transcriptional repressor Nrg1 (a DNA-binding protein involved in filamentous growth) via the *gul-1* homologue Ssd1 (Lee et al. 2015). If GUL-1 functions as a translational repressor in *N. crassa* (as it does in yeast) and is hyperactive when COT-1 is nonfunctional, one expected cellular response could be an increase in some of the cell wall remodeling gene transcription rates. This increase could stoichiometrically overcome the natural transcript-GUL-1 ratio and result in increased accumulation of cell wall carbohydrates.

Some of the results obtained concerning changes in transcript abundance are supported by details of the phenotypic suppression of the *cot-1* (ts) phenotype by deletion of *gul-1*. The Δ*gul-1*;*cot-1* (ts) double mutant produce septa and cell wall morphology which highly resemble that of the wild type, in marked contrast to the *cot-1* (ts) strain. The morphological observations are further supported by carbohydrate cell wall composition analysis showing the curbing of excess *cot-1* (ts) chitin content in a Δ*gul-1* background (as well as by the hyper sensitivity of Δ*gul-1* to Nikkomycin Z). Evidence for changes in sensitivity to chitin synthesis inhibitors in *gul-1* homologue mutants has also been reported in *C. albicans* (Song et al. 2008) and *C. lagenarium* (Tanaka et al. 2007).

Surprisingly, the changes in glucan were much lower. Changes in total glucan content may not reflect deviation in the proportions of β-1,3, mixed β-1,3/1,4-glucan and α-1,3-glucan linkages (which were not obtained in the current analysis). These may differentially contribute to cell wall architecture on the basis of arrangement (rather than quantity of the monomeric constituent).

We have demonstrated the presence of a mechanistic link between COT-1, GUL-1 and cell wall remodeling in *N. crassa*. Similar interactions are likely to be present in other filamentous fungi. Additional GUL-1-interacting partners have yet to be identified and in what manner the COT-1-GUL-1 pathway may also be involved in cell wall remodeling gene mRNA localization and temporal translation has yet to be determined.

Acknowledgments This work was supported by the Israel Science Foundation. The carbohydrate analysis was supported in part by the Department of Energy-funded Center for Plant and Microbial Complex Carbohydrates (DE-FG09- 93ER-20097). We thank Dr. Yael Friedmann for her assistance in the TEM analysis.

References

Adams DJ (2004) Fungal cell wall chitinases and glucanases. Microbiology 150:2029–2035

Baker LG, Specht CA, Lodge JK (2009) Chitinases are essential for sexual development but not vegetative growth in *Cryptococcus neoformans*. Eukaryot Cell 8:1692–1705

Beauvais A, Latge JP (2001) Membrane and cell wall targets in *Aspergillus fumigatus*. Drug Resist Updat 4:38–49

Beth Din A, Yarden O (1994) The *Neurospora crassa chs-2* gene encodes a nonessential chitin synthase. Microbiology 140:2189–2197

Beth Din A, Specht CA, Robbins PW, Yarden O (1996) *chs-4*, a class IV chitin synthase gene from *Neurospora crassa*. Mol Gen Genet 250:214–222

Bidlingmaier S, Weiss EL, Seidel C, Drubin DG, Snyder M (2001) The Cbk1p pathway is important for polarized cell growth and cell separation in *Saccharomyces cerevisiae*. Mol Cell Biol 21:2449–2462

Borkovich KA et al (2004) Lessons from the genome sequence of *Neurospora crassa*: tracing the path from genomic blueprint to multicellular organism. Microbiol Mol Biol Rev 68:1–108

Bowman SM, Free SJ (2006) The structure and synthesis of the fungal cell wall. BioEssays 28:799–808

Buhr TL, Oved S, Truesdell GM, Huang C, Yarden O, Dickman MB (1996) TB3, a glutamine-rich kinase-encoding gene from *Colletotrichum trifolii* complements the cot-1 mutant of *Neurospora crassa*. Mol Gen Genet 251:565–572

Damveld RA, Arentshorst M, Franken A, vanKuyk PA, Klis FM, van den Hondel C, Ram AFJ (2005) The *Aspergillus niger* MADS-box transcription factor RlmA is required for cell wall reinforcement in response to cell wall stress. Mol Microbiol 58:305–319

Davis RH (2000) Neurospora: contributions of a model organism. Oxford University, Oxford, pp 283–316

Delgado-Silva Y et al (2014) Participation of *Candida albicans* transcription factor RLM1 in cell wall biogenesis and virulence. PLoS One 9(1):e86270. doi:10.1371/journal.pone.0086270

Dodou E, Treisman R (1997) The *Saccharomyces cerevisiae* MADS-box transcription factor Rlm1 is a target for the Mpk1 mitogen-activated protein kinase pathway. Mol Cell Biol 17:1848–1859

Dunkler A, Walther A, Specht CA, Wendland J (2005) *Candida albicans* CHT3 encodes the functional homolog of the Cts1 chitinase of *Saccharomyces cerevisiae*. Fungal Genet Biol 42:935–947

Dunlap JC et al (2007) Enabling a community to dissect an organism: overview of the *Neurospora* functional genomics project. Adv Genet 57:49–96

Durrenberger F, Kronstad J (1999) The *ukc1* gene encodes a protein kinase involved in morphogenesis, pathogenicity and pigment formation in *Ustilago maydis*. Mol Gen Genet 261:281–289

Fajardo-Somera RA, Jöhnk B, Bayram Ö, Valerius O, Braus GH, Riquelme M (2015) Dissecting the function of the different chitin synthases in vegetative growth and sexual development in *Neurospora crassa*. Fungal Genet Biol 75:30–45

Free SJ (2013) Fungal cell wall organization and biosynthesis. Adv Genet 81:33–82

Fuchs BB, Mylonakis E (2009) Our paths might cross: the role of the fungal cell wall integrity pathway in stress response and cross talk with other stress response pathways. Eukaryot Cell 8:1616–1625

Fujioka T et al (2007) MpkA-dependent and -independent cell wall integrity signaling in *Aspergillus nidulans*. Eukaryot Cell 6:1497–1510

Fujiwara M, Horiuchi H, Ohta A, Takagi M (1997) A novel fungal gene encoding chitin synthase with a myosin motor-like domain. Biochem Biophys Res Commun 236:75–78

Gorovits R, Yarden O (2003) Environmental suppression of *Neurospora crassa cot-1* hyperbranching: a link between COT1 kinase and stress sensing. Eukaryot Cell 2:699–707

Gorovits R, Propheta O, Kolot M, Dombradi V, Yarden O (1999) A mutation within the catalytic domain of COT 1 kinase confers changes in the presence of two COT 1 isoforms and in Ser/Thr protein kinase and phosphatase activities in *Neurospora crassa*. Fungal Genet Biol 27:264–274

Gorovits R, Sjollema KA, Sietsma JH, Yarden O (2000) Cellular distribution of COT1 kinase in *Neurospora crassa*. Fungal Genet Biol 30:63–70

Hall RA (2015) Dressed to impress: impact of environmental adaptation on the *Candida albicans* cell wall. Mol Microbiol 97:7–17

Hanks SK, Hunter T (1995) Protein kinases 6. The eukaryotic protein-kinase superfamily: kinase (catalytic) domain structure and classification. FASEB J 9:576–596

Heiss C, Klutts JS, Wang Z, Doering TL, Azadi P (2009) The structure of *Cryptococcus neoformans* galactoxylomannan contains beta-D-glucuronic acid. Carbohydr Res 344:915–920

Hergovich A, Stegert MR, Schmitz D, Hemmings BA (2006) NDR kinases regulate essential cell processes from yeast to humans. Nat Rev Mol Cell Biol 7:253–264

Hogan DJ, Riordan DP, Gerber AP, Herschlag D, Brown PO (2008) Diverse RNA-binding proteins interact with functionally related sets of RNAs, suggesting an extensive regulatory system. PLoS Biol 6:2297–2313

Hurtado-Guerrero R, van Aalten DMF (2007) Structure of *Saccharomyces cerevisiae* chitinase 1 and screening-based discovery of potent inhibitors. Chem Biol 14:589–599

Jansen JM, Wanless AG, Seidel CW, Weiss EL (2009) Cbk1 regulation of the RNA-binding protein Ssd1 integrates cell fate with translational control. Curr Biol 19:2114–2120

Jaques AK et al (2003) Disruption of the gene encoding the ChiB1 chitinase of *Aspergillus fumigatus* and characterization of a recombinant gene product. Microbiology 149:2931–2939

Jimenez-Ortigosa C et al (2012) Chitin synthases with a myosin motor-like domain control the resistance of *Aspergillus*

fumigatus to echinocandins. Antimicrob Agents Chemother 56:6121–6131
Johns SA, Leeder AC, Safaie M, Turner G (2006) Depletion of *Aspergillus nidulans cot*A causes a severe polarity defect which is not suppressed by the nuclear migration mutation *nud*A2. Mol Genet Genom 275:593–604
Jung US, Levin DE (1999) Genome-wide analysis of gene expression regulated by the yeast cell wall integrity signalling pathway. Mol Microbiol 34:1049–1057
Karlsson M, Stenlid J (2008) Comparative evolutionary histories of the fungal chitinase gene family reveal non-random size expansions and contractions due to adaptive natural selection. Evol Bioinform Online 4:47–60
Koch L, Lodin A, Herold I, Ilan M, Carmeli S, Yarden O (2014) Sensitivity of *Neurospora crassa* to a marine-derived *Aspergillus tubingensis* anhydride exhibiting antifungal activity that is mediated by the MAS1 protein. Mar Drugs 12:4713–4731
Kong L-A et al (2012) Different chitin synthase genes are required for various developmental and plant infection processes in the rice blast fungus *Magnaporthe oryzae*. PLoS Pathog 8(2):e1002526. doi:10.1371/journal.ppat.1002526
Kottom TJ, Limper AH (2004) *Pneumocystis carinii* cell wall biosynthesis kinase gene CBK1 is an environmentally responsive gene that complements cell wall defects of cbk-deficient yeast. Infect Immun 72:4628–4636
Kovacs Z et al (2013) Effect of cell wall integrity stress and RlmA transcription factor on asexual development and autolysis in *Aspergillus nidulans*. Fungal Genet Biol 54:1–14
Kuranda MJ, Robbins PW (1991) Chitinase is required for cell separation during growth of *Saccharomyces cerevisiae*. J Biol Chem 266:19758–19767
Kurischko C, Kim HK, Kuravi VK, Pratzka J, Luca FC (2011) The yeast Cbk1 kinase regulates mRNA localization via the mRNA-binding protein Ssd1. J Cell Biol 192:583–598. doi:10.1083/jcb.201011061
Lee H-J, Kim J-M, Kang WK, Yang H, Kim J-Y (2015) The NDR kinase Cbk1 downregulates the transcriptional repressor Nrg1 through the mRNA-binding protein Ssd1 in *Candida albicans*. Eukaryot Cell 14:671–683
Levin DE (2011) Regulation of cell wall biogenesis in *Saccharomyces cerevisiae*: the cell wall integrity signaling pathway. Genetics 189:1145–1175
Li M, Jiang C, Wang Q, Zhao Z, Jin Q, Xu J-R, Liu H (2016) Evolution and functional insights of different ancestral orthologous clades of chitin synthase genes in the fungal tree of life. Front Plant Sci 7:37. doi:10.3389/fpls.2016.00037
Maddi A, Free SJ (2010) α-1,6-Mannosylation of N-linked oligosaccharide present on cell wall proteins is required for their incorporation into the cell wall in the filamentous fungus *Neurospora crassa*. Eukaryot Cell 9:1766–1775
Maddi A, Bowman SM, Free SJ (2009) Trifluoromethanesulfonic acid-based proteomic analysis of cell wall and secreted proteins of the ascomycetous fungi *Neurospora crassa* and *Candida albicans*. Fungal Genet Biol 46:768–781
Maerz S et al (2008) The nuclear Dbf2-related kinase COT1 and the mitogen-activated protein kinases MAK1 and MAK2 genetically interact to regulate filamentous growth, hyphal fusion and sexual development in *Neurospora crassa*. Genetics 179:1313–1325
Mamarabadi M, Jensen B, Jensen DF, Luebeck M (2008) Real-time RT-PCR expression analysis of chitinase and endoglucanase genes in the three-way interaction between the biocontrol strain *Clonostachys rosea* IK726, *Botrytis cinerea* and strawberry. FEMS Microbiol Lett 285:101–110
McNemar MD, Fonzi WA (2002) Conserved serine/threonine kinase encoded by CBK1 regulates expression of several hypha associated transcripts and genes encoding cell wall proteins in *Candida albicans*. J Bacteriol 184:2058–2061
Motoyama T, Fujiwara M, Kojima N, Horiuchi H, Ohta A, Takagi M (1996) The *Aspergillus nidulans* genes *chs*A and *chs*D encode chitin syntheses which have redundant functions in conidia formation. Mol Gen Genet 251:442–450
Osherov N, Yarden O (2010) The cell wall of filamentous fungi. In: Borkovich KA, Ebbole DJ (eds) Cellular and molecular biology of filamentous fungi. ASM, Washington, DC, pp 224–237
Pearce LR, Komander D, Alessi DR (2010) The nuts and bolts of AGC protein kinases. Nat Rev Mol Cell Biol 11:9–22
Perkins D, Radford A, Newmeyer D, Bjorkman M (1982) Comosomal loci of *Neurospora crassa*. Microbiol Rev 46:426–570
Racki WJ, Becam AM, Nasr F, Herbert CJ (2000) Cbk1p, a protein similar to the human myotonic dystrophy kinase, is essential for normal morphogenesis in *Saccharomyces cerevisiae*. EMBO J 19:4524–4532
Riquelme M, Bartnicki-Garcia S (2008) Advances in understanding hyphal morphogenesis: ontogeny, phylogeny and cellular localization of chitin synthases. Fungal Biol Rev 22:56–70
Riquelme M et al (2011) Architecture and development of the *Neurospora crassa* hypha—a model cell for polarized growth. Fungal Biol 115:446–474
Sanchez-Leon E, Verdin J, Freitag M, Roberson RW, Bartnicki-Garcia S, Riquelme M (2011) Traffic of chitin synthase 1 (CHS-1) to the Spitzenkörper and developing septa in hyphae of *Neurospora crassa*: actin dependence and evidence of distinct microvesicle populations. Eukaryot Cell 10:683–695
Scheffer J, Ziv C, Yarden O, Tudzynski P (2005) The COT1 homologue CPCOT1 regulates polar growth and branching and is essential for pathogenicity in *Claviceps purpurea*. Fungal Genet Biol 42:107–118
Seidl V (2008) Chitinases of filamentous fungi: a large group of diverse proteins with multiple physiological functions. Fungal Biol Rev 22:36–42
Seiler S, Vogt N, Ziv C, Gorovits R, Yarden O (2006) The STE20/germinal center kinase POD6 interacts with the NDR kinase COT1 and is involved in polar tip extension in *Neurospora crassa*. Mol Biol Cell 17:4080–4092
Song Y et al (2008) Role of the RAM network in cell polarity and hyphal morphogenesis in *Candida albicans*. Mol Biol Cell 19:5456–5477
Takeshita N, Yamashita S, Ohta A, Horiuchi H (2006) *Aspergillus nidulans* class V and VI chitin synthases CsmA and CsmB, each with a myosin motor-like domain, perform compensatory functions that are essential for hyphal tip growth. Mol Microbiol 59:1380–1394
Tanaka S et al (2007) *Saccharomyces cerevisiae* SSD1 orthologues are essential for host infection by the ascomycete plant pathogens *Colletotrichum lagenarium* and *Magnaporthe grisea*. Mol Microbiol 64:1332–1349
Tentler S et al (1997) Inhibition of *Neurospora crassa* growth by a glucan synthase-1 antisense construct. Curr Microbiol 34:303–308
Teparic R, Mrsa V (2013) Proteins involved in building, maintaining and remodeling of yeast cell walls. Curr Genet 59:171–185
Terenzi HF, Reissig JL (1967) Modifiers of the *cot* gene in *Neurospora*: the gulliver mutants. Genetics 56:321–329
Thompson JR et al (1999) A glucan synthase FKS1 homolog in *Cryptococcus neoformans* is single copy and encodes an essential function. J Bacteriol 181:444–453
Tzelepis GD, Melin P, Jensen DF, Stenlid J, Karlsson M (2012) Functional analysis of glycoside hydrolase family 18 and 20 genes in *Neurospora crassa*. Fungal Genet Biol 49:717–730
Uesono Y, Tohe A, Kikuchi Y (1997) Ssd1p of *Saccharomyces cerevisiae* associates with RNA. J Biol Chem 272:16103–16109

Valiante V, Macheleidt J, Foege M, Brakhage AA (2015) The *Aspergillus fumigatus* cell wall integrity signaling pathway: drug target, compensatory pathways, and virulence. Front Microbiol 6:325. doi:10.3389/fmicb.2015.00325

Wanless AG, Lin Y, Weiss EL (2014) Cell morphogenesis proteins are translationally controlled through UTRs by the Ndr/LATS target Ssd1. PLoS One 9(1):e85212. doi:10.1371/journal.pone

Watanabe Y, Takaesu G, Hagiwara M, Irie K, Matsumoto K (1997) Characterization of a serum response factor-like protein in *Saccharomyces cerevisiae*, Rlm1, which has transcriptional activity regulated by the Mpk1 (Slt2) mitogen-activated protein kinase pathway. Mol Cell Biol 17:2615–2623

Yamazaki H, Tanaka A, J-i Kaneko, Ohta A, Horiuchi H (2008) *Aspergillus nidulans* ChiA is a glycosylphosphatidylinositol (GPI)-anchored chitinase specifically localized at polarized growth sites. Fungal Genet Biol 45:963–972

Yarden O, Plamann M, Ebbole DJ, Yanofsky C (1992) *cot-1*, a gene required for hyphal elongation in *Neurospora crassa*, encodes a protein kinase. EMBO J 11:2159–2166

Ziv C, Kra-Oz G, Gorovits R, Maerz S, Seiler S, Yarden O (2009) Cell elongation and branching are regulated by differential phosphorylation states of the nuclear Dbf2-related kinase COT1 in *Neurospora crassa*. Mol Microbiol 74:974–989