Curr Genet (2016) 62:185–189
DOI 10.1007/s00294-015-0515-9

ORIGINAL ARTICLE

Distinct cell cycle regulation during saprophytic and pathogenic growth in fungal pathogens

Cong Jiang[1,2] · Jin-Rong Xu[2] · Huiquan Liu[1]

Received: 18 August 2015 / Revised: 20 August 2015 / Accepted: 22 August 2015 / Published online: 4 September 2015

Abstract In a number of dimorphic and hemibiotrophic pathogens, cell cycle regulation has been shown to be important for morphological changes related to infectious growth or infection-related morphogenesis. However, the role of mitotic CDK kinase Cdc2, the key regulator of cell cycle, in pathogenic growth is not clear, because most fungal pathogens have a single *CDC2* gene that is essential for cell cycle progression and viability. Interestingly, the wheat scab fungus *Fusarium graminearum* has two *CDC2* genes. Although *CDC2A* and *CDC2B* have redundant functions in vegetative growth and asexual production, only *CDC2A* is required for invasive growth and plant infection. In this study, we showed that Cdc2A and Cdc2B interacted with each other and may form homo- and heterodimers in vegetative hyphae. We also identified sequence and structural differences between Cdc2A and Cdc2B that may be related to their functional divergence. These results, together with earlier studies with cyclins, important for differentiation and infection in *Candida albicans and Ustilago maydis*, indicated that dimorphic and hemibiotrophic fungal pathogens may have stage-specific cyclin–CDK combinations or CDK targets during saprophytic and pathogenic growth.

Keywords Gibberella zeae · Fusarium head blight · CDK · *CDC2* · CDC28 · Cyclin

Communicated by M. Kupiec.

✉ Huiquan Liu
liuhuiquan@nwsuaf.edu.cn

[1] State Key Laboratory of Crop Stress Biology for Arid Areas, College of Plant Protection, Northwest A&F University, Yangling, Shaanxi, China

[2] Department of Botany and Plant Pathology, Purdue University, West Lafayette, IN, USA

Introduction

In eukaryotic organisms, cell cycle involves a series of complex cellular events, including DNA synthesis and chromosomes replication (Sendinc et al. 2015). The cyclin-dependent protein kinases (CDKs) function as the central regulator of cell cycle and their stage-specific activities are determined by interacting with different cyclins (Bloom and Cross 2007). In addition, CDKs are subjected to activation phosphorylation by CDK kinase and inhibitory phosphorylation by Wee1 kinase for cell cycle progression (Morgan 1997). In the budding and fission yeasts that are used as the model organisms for studying cell cycle and other cellular events (Hapala et al. 2013; Teparic and Mrsa 2013; Veide Vilg et al. 2014), a single CDK known as Cdc2 or Cdc28 is essential for cell cycle progression (Fisher and Nurse 1996; Mendenhall and Hodge 1998). Similarly, the model filamentous fungi *Neurospora crassa* and *Aspergillus nidulans* have a single copy Cdc2 ortholog that is essential for hyphal growth (Borkovich et al. 2004; Osmani et al. 1994).

Unlike the model yeasts and filamentous fungi, the wheat scab fungus *Fusarium graminearum* has two CDK genes, *CDC2A* and *CDC2B* (Wang et al. 2011). Deletion of either *CDC2A* or *CDC2B* had no obvious defects in vegetative growth and conidiation, but deletion of both is lethal, indicating that these two *CDC2* orthologs have redundant functions during hyphal growth and asexual reproduction (Liu et al. 2015). However, it appears that their functions have diverged and only *CDC2A* is important for plant infection and sexual reproduction. In infection assays with flowing wheat heads and corn silks, the *cdc2B* mutant was normal in virulence, but the *cdc2A* mutant was almost non-pathogenetic. Further characterization showed that the *cdc2A* mutant formed penetration structures, but failed to develop

lobed infectious hyphae inside wheat coleoptile cells (Liu et al. 2015). During sexual reproduction, the *cdc2B* mutant was normal in ascospore formation, but the *cdc2A* mutant was defective in ascosporogenesis (Liu et al. 2015). These observations indicate that Cdc2A and Cdc2B, two closely related Cdc2 paralogs, have distinct functions in different developmental and infection stages in *F. graminearum*. Although similar observation has not been reported in other fungi and animals with a single mitotic CDK, the B-type CDKs in plants are known to play development-specific roles in cell cycle regulation (Harashima et al. 2013).

Materials and methods

The interaction between Cdc2A and Cdc2B was assayed by yeast two-hybrid and co-IP as described (Liu et al. 2015). Briefly, for yeast two-hybrid assays, the ORFs of *CDC2A* and *CDC2B* were amplified from cDNA of PH-1 and cloned into pGBKT7 as the bait construct and pGADT7 as the prey construct, respectively. The resulting *CDC2A*-bait and *CDC2B*-prey constructs were co-transformed into yeast strain AH109 (Clontech). The resulting transformants were assayed for growth on synthetic dropout (SD) medium lacking tryptophan, leucine, and histidine (SD-Trp-Leu-His) and β-galactosidase activities. For co-IP assays, the *CDC2A*–GFP and *CDC2B*–3xFLAG fusion constructs were generated by cloning genomic fragments containing these two genes into pFL2 and PFL7 vectors (Wang et al. 2011; Liu et al. 2015), respectively. The resulting fusion constructs were co-transformed into PH-1. The expression of both transforming vectors was confirmed by PCR and Western blot analysis. Total proteins isolated from transformants expressing both transforming constructs were incubated with anti-Flag M2 beads (Sigma-Aldrich, St. Louis, MO). Proteins bound to anti-FLAG beads were eluted following the instructions provided by Sigma-Aldrich. Western blots of total proteins and proteins eluted from anti-Flag M2 beads were detected with the anti-GFP (Roche, Indianapolis, IN) and anti-actin (Sigma-Aldrich) antibodies.

Results and discussion

To further determine the functional relationship between Cdc2A and Cdc2B, we first examined their interactions by yeast two-hybrid assays. Yeast cells expressing the Cdc2A bait and Cdc2B prey constructs were able to growth on SD-Leu-Trp-His plates and had beta-galactosidase activities (Fig. 1a), indicating that they may directly interact with each other. To confirm this observation, we also generated *CDC2A*–GFP and *CDC2B*–3xFLAG constructs and co-transformed them into the wild-type strain PH-1. In the resulting transformants, the expression of Cdc2A–GFP and Cdc2B–3xFLAG fusion proteins was confirmed by Western blot analysis with total proteins isolated from vegetative hyphae. In proteins of the same transformants eluted from anti-FLAG beads, the Cdc2A–GFP band was detected with an anti-GFP antibody (Fig. 1b). These results showed that Cdc2A and Cdc2B interact with each other and may form heterodimers in vegetative hyphae. The formation of heterodimers between Cdc2 proteins has not been reported, likely due to the fact that the model organisms studied for cell cycle regulation has a single Cdc2. Therefore, it will be important to further characterize the roles of possible homo- and heterodimers of Cdc2 paralogs in cell cycle regulation in *F. graminearum*.

Cdc2A and Cdc2B are 78 % identical in amino acid sequences. Both of them have typical CDK structures, including the inhibitory and activation phosphorylation sites, activation T-loop, and cyclin-binding site PSTAIRE (Liu et al. 2015; Morgan 1997). They also have similar 3-D structures after protein structure prediction (Liu et al. 2015). However, Cdc2A and Cdc2B have some sequence and structural differences that may be related to their functional divergence (Fig. 1c). Seven residues in the 55–68 amino acid region adjacent to the PSTAIRE helix (cyclin-binding site) differ between these two Cdc2 proteins, which may affect the interaction of Cdc2A/2B with CDK cyclins. Cdc2A and Cdc2B also are different at residue 194 in the T-loop that may affect its activation. In addition, based on their predicted 3D structures, the surface topography and the width of the active cleft site in Cdc2A and Cdc2B also are slightly different (Fig. 1c). Some of these sequence or structure features may be responsible for the functional specificity of Cdc2A, and characterization of their functions will be important to better understand cell cycle regulation during invasive growth in *F. graminearum*.

To our knowledge, Cdc2A is the first CDK that has been shown to be specifically required for infectious growth. This is not surprising because most fungal pathogens have only one *CDC2* ortholog and the single copy *CDC2* gene is essential for viability. However, it has been well documented in dimorphic pathogens such as the human pathogen *Candida albicans* and corn smut fungus *U. maydis* that cell cycle regulation plays an important role in pathogenesis and morphogenesis, although they have only one Cdc2 ortholog (Perez-Martin and Castillo-Lluva 2008; Perez-Martin et al. 2006; Wilson and Hube 2010; Zheng, et al. 2004). In *C. albicans*, the yeast-to-hypha transition is related to pathogenesis. A hyphal-specific cyclin Hgc1 (hypha-specific G1 cyclin) has been identified and is required for cell cycle progression in hyphae and during infection (Wilson and Hube 2010; Zheng et al. 2004).

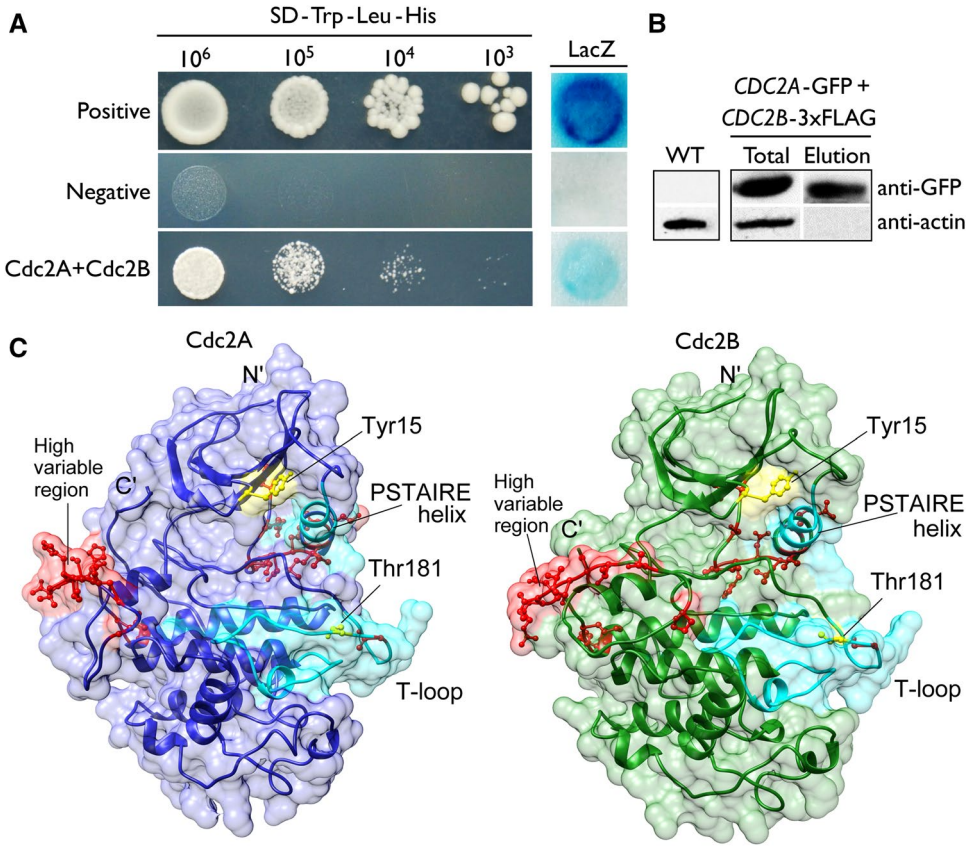

Fig. 1 Two Cdc2 orthologs and their functional relationship in *F. graminearum*. **a** Different concentrations of yeast cells (cells/ml) of the transformant expressing the Cdc2A-bait and Cdc2B-prey constructs were assayed for growth on SD-Leu-Trp-His plates and for beta-galactosidase (LacZ) activities. The positive and negative controls were provided by the Matchmaker yeast two-hybrid library construct kit. **b** Immunoblots of total proteins (Total) and proteins eluted from anti-FLAG M2 beads (Elution) of the transformant expressing both Cdc2A–GFP and Cdc2B–3xFLAG fusion constructs were detected with an anti-actin or anti-GFP antibody. Total proteins isolated from the wild-type (WT) PH-1 were included as the control. **c** Predicted tertiary structures of Cdc2A and Cdc2B. The N'- and C'-termini, PSTAIRE helix, activation loop (T-loop), and inhibitory (Tyr15) and activation (Thr181) phosphorylation sites are labeled. The atoms and bonds of residues different between Cdc2A and Cdc2B in the T-loop, in a high variable region (106-123 aa) and in the region (55-68 aa) adjacent to the cyclin-binding sites, are shown and marked in *red*

Hgc1 is important for hyphal tip growth and septum formation (Cao et al. 2006; Carlisle and Kadosh 2010; Wang 2009).

In *U. maydis*, the haploid yeast form is saprophytic. Mating between compatible yeast cells gives rise to the dikaryotic hyphal form that is obligate pathogenic and requires an intricate cell division process to coordinate mitosis of two compatible nuclei. The single *cdk1* CDK gene is essential and inhibitory phosphorylation of Cdk1 is required for conjugation tube formation and plant penetration (Mielnichuk et al. 2009; Sgarlata and Perez-Martin 2005). Among the Cdk1 cyclin genes that have been characterized in *U. maydis*, the G1 cyclin Cln1 regulates cell size and sexual development (Castillo-Lluva and Perez-Martin 2005). Whereas Clb1 cyclin is required for G1/S and G2/M transitions, Clb2 cyclin regulates morphogenesis and plant infection (Garcia-Muse et al. 2004).

In the hemibiotrophic rice blast fungus *Magnaporthe oryzae*, infectious hyphae are bulbous and have pseudohyphal growth (Mosquera et al. 2009; Zhou et al. 2012), suggesting the involvement of infection-specific cell cycle regulation although the underlying mechanism is not clear. Nevertheless, the relationship between cell cycle and appressorium formation has been characterized. One round of mitosis in germ tubes is required for appressorium formation and nuclei in appressoria are arrested in G1 (Caracuel-Rios and Talbot 2007; Saunders et al. 2010b). Appressorium development also requires spatial uncoupling of mitosis and cytokinesis (Saunders et al. 2010a). Furthermore, regulation of Rho GTPases by the *MoPPG1* protein phosphatase 2A gene is required for plant cell penetration (Du et al. 2013), and biotrophic growth in rice cells is governed by a transketolase-mediated *in planta*-specific metabolic checkpoint that regulates cell cycle (Fernandez

et al. 2014). Recently, we have showed that the MoGti1 transcription factor may function as a stage-specific target of CDK during invasive growth (our unpublished data).

Unfortunately, in other plant pathogenic fungi, cell cycle regulation is rarely studied and our understanding of the coordination between cell cycle and pathogenesis is limited. However, similar to *M. oryzae*, many hemibiotrophic pathogens have different morphology and physiological properties between saprophytic and infectious hyphae. They will need the reset of cell cycle or proper cell cycle regulation for the formation of various infection structures and biotrophic infectious hyphae. Although these fungal pathogens may have only a single *CDC2* gene, like *C. albicans* (Zheng and Wang 2004) and *U. maydis*, they may use stage-specific cyclins to regulate infectious growth. Even in fungi like *F. graminearum* with two Cdc2 orthologs, it remains possible that they bind with different cyclins and play distinct roles during plant infection. Unfortunately, cyclins are not well characterized in filamentous ascomycetes.

Besides binding to cyclins, the activation phosphorylation by CDK-activating kinase (CAK) and inhibitory phosphorylation by Wee1 kinase also are important for regulating CDK activities (Morgan 1997). *F. graminearum* and all the filamentous ascomycetes we examined have a single copy CAK and Wee1 kinase genes. Therefore, it is unlikely for these fungi to use different CAK or Wee1 kinases to differentially regulate infectious and saprophytic growth. Nevertheless, deletion of *CAK1* was not lethal, but the *Fgcak1* mutant was non-pathogenic in *F. graminearum* (Liu et al. 2015), indicating that FgCak1 is essential for the cell cycle regulation during plant infection but not in vegetative hyphae. The *Fgwee1* deletion mutant also was defective in plant infection (Wang et al. 2011), suggesting a possible role of FgWee1 in the functional specificity of Cdc2A. In *U. maydis*, the inhibitory phosphorylation of Cdk1 is important for plant penetration (Sgarlata and Perez-Martin 2005).

Overall, studies on *C. albicans, U. maydis,* and *F. graminearum* suggest that cell cycle regulation may be different during saprophytic and pathogenic growth. This is likely to be true for many other dimorphic or hemibiotrophic fungal pathogens that have different hyphal morphology between vegetative and infectious growth, and possibly have stage-specific cyclin–CDK combinations or CDK targets. This hypothesis may be also applicable to obligate pathogens, such as powdery mildew and rust fungi. Based on the genomes that have been sequenced, obligate pathogens have most of the genes required for primary metabolism and growth (Duplessis et al. 2011; Fellers et al. 2013; Spanu et al. 2010; Zheng et al. 2014). The reason for obligate parasitism in rust and powdery mildew fungi could not be simply related to the gene contents of their genomes. It is possible that obligate pathogens may have lost cell cycle-related genes required for saprophytic growth during evolution, but retained the cell cycle progression machinery specific for infectious growth, resulting in the inability to grow on synthetic media.

Acknowledgments We apologize to those authors whose contributions could not be cited due to space limitation. This work was supported by the National Major Project of Breeding for New Transgenic Organisms (2012ZX08009003).

References

Bloom J, Cross FR (2007) Multiple levels of cyclin specificity in cell-cycle control. Nat Rev Mol Cell Biol 8:149–160. doi:10.1038/nrm2105

Borkovich KA, Alex LA, Yarden O, Freitag M, Turner GE, Read ND, Seiler S, Bell-Pedersen D, Paietta J, Plesofsky N, Plamann M, Goodrich-Tanrikulu M, Schulte U, Mannhaupt G, Nargang FE, Radford A, Selitrennikoff C, Galagan JE, Dunlap JC, Loros JJ, Catcheside D, Inoue H, Aramayo R, Polymenis M, Selker EU, Sachs MS, Marzluf GA, Paulsen I, Davis R, Ebbole DJ, Zelter A, Kalkman ER, O'Rourke R, Bowring F, Yeadon J, Ishii C, Suzuki K, Sakai W, Pratt R (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. doi:10.1128/MMBR.68.1.1-108.2004

Cao F, Lane S, Raniga PP, Lu Y, Zhou Z, Ramon K, Chen JY, Liu HP (2006) The Flo8 transcription factor is essential for hyphal development and virulence in *Candida albicans*. Mol Biol Cell 17:295–307. doi:10.1091/mbc.E05-06-0502

Caracuel-Rios Z, Talbot NJ (2007) Cellular differentiation and host invasion by the rice blast fungus *Magnaporthe grisea*. Curr Opin Microbiol 10:339–345. doi:10.1016/j.mib.2007.05.019

Carlisle PL, Kadosh D (2010) *Candida albicans* Ume6, a filament-specific transcriptional regulator, directs hyphal growth via a pathway involving hgc1 cyclin-related protein. Eukaryot Cell 9:1320–1328. doi:10.1128/ec.00046-10

Castillo-Lluva S, Perez-Martin J (2005) The induction of the mating program in the phytopathogen *Ustilago maydis* is controlled by a G1 cyclin. Plant Cell 17:3544–3560. doi:10.1105/tpc.105.036319

Du Y, Shi Y, Yang J, Chen X, Xue M, Zhou W, Peng YL (2013) A serine/threonine-protein phosphatase PP2A catalytic subunit is essential for asexual development and plant infection in *Magnaporthe oryzae*. Curr Genet 59:33–41. doi:10.1007/s00294-012-0385-3

Duplessis S, Cuomo CA et al (2011) Obligate biotrophy features unraveled by the genomic analysis of rust fungi. Proc Nat Acad Sci USA 108:9166–9171. doi:10.1073/pnas.1019315108

Fellers JP, Soltani BM, Bruce M, Linning R, Cuomo CA, Szabo LJ, Bakkeren G (2013) Conserved loci of leaf and stem rust fungi of wheat share synteny interrupted by lineage-specific influx of repeat elements. BMC Genomics 14: CABI:20133077744 doi:10.1186/1471-2164-14-60

Fernandez J, Marroquin-Guzman M, Wilson RA (2014) Evidence for a transketolase-mediated metabolic checkpoint governing biotrophic growth in rice cells by the blast fungus *Magnaporthe oryzae*. PLoS Pathog 10:e1004354. doi:10.1371/journal.ppat.1004354

Fisher DL, Nurse P (1996) A single fission yeast mitotic cyclin $B^{p34(cdc2)}$ kinase promotes both S-phase and mitosis in the absence of G(1) cyclins. EMBO J 15:850–860

Garcia-Muse T, Steinberg G, Perez-Martin J (2004) Characterization of B-type cyclins in the smut fungus *Ustilago maydis*: roles in morphogenesis and pathogenicity. J Cell Sci 117:487–506. doi:10.1242/jcs.00877

Hapala I, Griac P, Nosek J, Sychrova H, Tomaska L (2013) Yeast membranes and cell wall: from basics to applications. Curr Genet 59:167–169. doi:10.1007/s00294-013-0408-8

Harashima H, Dissmeyer N, Schnittger A (2013) Cell cycle control across the eukaryotic kingdom. Trends Cell Biol 23:345–356. doi:10.1016/j.tcb.2013.03.002

Liu H, Zhang S, Ma J, Dai Y, Li C, Lyu X, Wang C, Xu JR (2015) Two Cdc2 Kinase Genes with Distinct Functions in Vegetative and Infectious Hyphae in *Fusarium graminearum*. PLoS Pathog 11:e1004913. doi:10.1371/journal.ppat.1004913

Mendenhall MD, Hodge AE (1998) Regulation of Cdc28 cyclin-dependent protein kinase activity during the cell cycle of the yeast *Saccharomyces cerevisiae*. Microbiol Mol Biol Rev 62:1191–1243

Mielnichuk N, Sgarlata C, Perez-Martin J (2009) A role for the DNA-damage checkpoint kinase Chk1 in the virulence program of the fungus *Ustilago maydis*. J Cell Sci 122:4130–4140. doi:10.1242/jcs.052233

Morgan DO (1997) Cyclin-dependent kinases: engines, clocks, and microprocessors. Annu Rev Cell Dev Biol 13:261–291. doi:10.1146/annurev.cellbio.13.1.261

Mosquera G, Giraldo MC, Khang CH, Coughlan S, Valent B (2009) Interaction transcriptome analysis identifies *Magnaporthe oryzae* BAS1-4 as biotrophy-associated secreted proteins in rice blast disease. Plant Cell 21:1273–1290. doi:10.1105/tpc.107.055228

Osmani AH, van Peij N, Mischke M, O'Connell MJ, Osmani SA (1994) A single $p34^{cdc2}$ protein kinase (encoded by nimXcdc2) is required at G1 and G2 in *Aspergillus nidulans*. J Cell Sci 107(Pt 6):1519–1528

Perez-Martin J, Castillo-Lluva S (2008) Connections between polar growth and cell cycle arrest during the induction of the virulence program in the phytopathogenic fungus *Ustilago maydis*. Plant signaling & behavior 3:480–481

Perez-Martin J, Castillo-Lluva S, Sgarlata C, Flor-Parra I, Mielnichuk N, Torreblanca J, Carbo N (2006) Pathocycles: *Ustilago maydis* as a model to study the relationships between cell cycle and virulence in pathogenic fungi. Mol Genet Genom 276:211–229. doi:10.1007/s00438-006-0152-6

Saunders DG, Dagdas YF, Talbot NJ (2010a) Spatial uncoupling of mitosis and cytokinesis during appressorium-mediated plant infection by the rice blast fungus *Magnaporthe oryzae*. Plant Cell 22:2417–2428. doi:10.1105/tpc.110.074492

Saunders DGO, Aves SJ, Talbot NJ (2010b) Cell cycle-mediated regulation of plant infection by the rice blast fungus. Plant Cell 22:497–507. doi:10.1105/tpc.109.072447

Sendinc E, Jambhekar A, Shi Y (2015) Remodeling your way out of cell cycle. Cell 162:237–238. doi:10.1016/j.cell.2015.06.050

Sgarlata C, Perez-Martin J (2005) Inhibitory phosphorylation of a mitotic cyclin-dependent kinase regulates the morphogenesis, cell size and virulence of the smut fungus *Ustilago maydis*. J Cell Sci 118:3607–3622. doi:10.1242/jcs.02499

Spanu PD, Abbott JC et al (2010) Genome expansion and gene loss in powdery mildew fungi reveal tradeoffs in extreme parasitism. Science 330:1543–1546. doi:10.1126/science.1194573

Teparic R, Mrsa V (2013) Proteins involved in building, maintaining and remodeling of yeast cell walls. Curr Genet 59:171–185. doi:10.1007/s00294-013-0403-0

Veide Vilg J, Dahal S, Ljungdahl T, Grotli M, Tamas MJ (2014) Application of a peptide-based assay to characterize inhibitors targeting protein kinases from yeast. Curr Genet 60:193–200. doi:10.1007/s00294-014-0424-3

Wang Y (2009) CDKs and the yeast-hyphal decision. Curr Opin Microbiol 12:644–649. doi:10.1016/j.mib.2009.09.002

Wang CF, Zhang SJ, Hou R, Zhao ZT, Zheng Q, Xu QJ, Zheng DW, Wang GH, Liu HQ, Gao XL, Ma JW, Kistler HC, Kang ZS, Xu JR (2011) Functional analysis of the kinome of the wheat scab fungus *Fusarium graminearum*. PLoS Pathog 7:e1002460. doi:10.1371/journal.ppat.1002460

Wilson D, Hube B (2010) Hgc1 mediates dynamic *Candida albicans*-endothelium adhesion events during circulation. Eukaryot Cell 9:278–287. doi:10.1128/ec.00307-09

Zheng X, Wang Y (2004) Hgc1, a novel hypha-specific G1 cyclin-related protein regulates *Candida albicans* hyphal morphogenesis. EMBO J 23:1845–1856. doi:10.1038/sj.emboj.7600195

Zheng W, Huang L et al (2014) High genome heterozygosity and endemic genetic recombination in the wheat stripe rust fungus. Nat Commun 4:2673. doi:10.1038/ncomms3673

Zhou X, Zhang H, Li G, Shaw B, Xu JR (2012) The cyclase-associated protein Cap1 is important for proper regulation of infection-related morphogenesis in *Magnaporthe oryzae*. PLoS Pathog 8:e1002911. doi:10.1371/journal.ppat.1002911