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Cover Letter

CONSEJO SUPERIOR
DE INVESTIGACIONES
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DE BIOTECNOLOGIA

Dr. José Pérez-Martín

Fungal Genetics and Biology

Editor

Date: May 19, 2008

Subject: Revision of a manuscript to *Fungal Genetics and Biology*

Dear Dr. Kronstad:

Please find enclosed a revised version of the manuscript entitled “14-3-3 regulates the G2/M transition in the basidiomycete *Ustilago maydis*" (FGB-08-83)

In this new version, we tried to address all reviewer´s comments (see attached letter). We addressed the question raised by reviewers 1 and 2 about Figure 2E, including a wild-type control. We followed the suggestions of both reviewers to improve the text and added explanations to clarify the questions of reviewers. We believe that now the work is clearer. We are confident that you will find interesting this new version and we want to thank you and the reviewers for the effort and interest in this work.

Sincerely

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ANSWERS TO REVIEWERS

Reviewer #1: In this manuscript, Mielnichuk and Perez-Martin present the functional characterization of the sole 14-3-3 homologue in Ustilago maydis, Bmh1. Because the deletion of bmh1 appears to be lethal, they generate a conditional promoter shut-off allele that reveals roles in G2 regulation and the control of cell morphology. They provide fairly compelling evidence that Bmh1 interacts with Cdc25 and regulates its cytoplasmic retention. They also describe a possible role for Bmh1 in the DNA damage response. They conclude that the Bmh1-Cdc25 regulatory module shares conserved cell cycle regulatory functions in U. maydis and the fission yeast Schizosaccharomyces pombe.

Despite their importance as signaling modulators based on studies in yeast and animals, little is actually known about the role of 14-3-3 proteins in filamentous fungi. The demonstration that they regulate the G2/M transition by modulating Cdc25 localization is notable, and although there are undoubtedly other relevant binding partners to be found, this manuscript represents a good starting point.

Prior to publication, the authors should address the following points;

1. It would be worthwhile including a description of the bmh1 deletion phenotype observed in the hygromycin-resistant inviable spores (page 11). This could presumably be observed on a dissecting microscope. If the spores exhibit abnormal morphology (i.e., they are round), this would support the view that Bmh1 has additional essential functions beyond regulating Cdc25.

The spores obtained after infection with the diploid strain carrying a deletion in one of the *bmh1* alleles showed a wild-type morphology. However, you have to keep in mind that *U. maydis* spores are diploid and that they enter into meiosis after germination. To determine the meiotic progeny we follow a "in masse" protocol after germination described by Holliday 1974.

2. Along the same lines as the previous point, did the 5-fold overexpression of Bmh1 shown in Fig. 2B cause any observable defect? The colonies appear normal, but it would be worthwhile commenting on whether the cells exhibit abnormal shapes or are elongated due to increased time in G2.

In spite of the higher level of expression of the *bmh1*^{crg1} allele in induction conditions compared to wild-type allele, we found no differences with respect to wild-type cells in morphology when mutant cells were grown in permissive liquid culture (YPA, Fig. 2D). We included a comment in the text stressing that we found no differences

3. Fig. 2E is extremely confusing. It is not clear which panels are wildtype, nor is it clear which shows cells after prolonged incubation at restrictive conditions.

All cells showed in the first version of Fig. 2E were mutant. We agree the reviewer in that it is confusing and in this new version we included a figure with wild-type cells.

4. For Fig. 3C, the authors should indicate how long the cells had been incubated at restrictive conditions (as a point of comparison to the cells shown in Fig. 2E).

In this figure cells were incubated for 6 hours in complete medium (CMD). We choose these conditions because at this time of incubation the morphology is not yet affected and the use of CMD medium produces a brighter Cdc25-GFP signal when compared with YPD (that produce a brighter background). We included in the legend these explanations.

5. The statement that "in fungi, cell cycle control is intimately linked to morphogenesis" (page 19) is somewhat misleading. While this is clearly true for yeasts and dimorphic fungi, it remains unclear how strongly morphogenesis (i.e., polarized hyphal growth) is linked to the cell cycle in filamentous fungi.

We changed this statement, focusing in yeast.

6. Have the authors tested whether over-expression of Cdc25 results in increased accumulation of Cdc25 in the nucleus (Discussion, page 20)? If it doesn't, this could explain why the bmh1 phenotype is more severe.

We already reported (Sgarlata and Pérez-Martin, 2005) that overexpression of Cdc25, even when affected the cell cycle and morphology was not lethal to the cell. In these overexpression conditions we found a strong accumulation of Cdc25 in the nucleus. We believe that the *bmh1* phenotype has to be explain not only as a function of the cell cycle effects but also as a consequence of the lack of Bmh1 in other morphogenetic processes, as it has been proposed in *C. albicans* and *S. cerevisiae*.

Reviewer #2: In this paper, Mielnichuk and Perez-Martin identify and characterize the 14-3-3 protein with regards to the cell cycle in Ustilago maydis. The interesting focus of this paper is the interpretation that fungal 14-3-3 proteins associate with critical stages in the cell cycle and that this differs among fungal species. They demonstrate by fusion proteins, co-IP, and IF that the Ustilago 14-3-3 plays a critical role in the G2 phase and associates with Cdc25p as seen in Schizosaccharomyces pombe (and mammalian cells). The data is sufficient and convincing. The entire paper would benefit by shortening the introduction and results section. The paper should also be proofread for English.

1. Abstract. It is not entirely correct to state that the role of 14-3-3 in cell cycle regulation is not conserved among fungal species. As the authors point out, the role of 14-3-3 is probably the same in that it regulates critical points in the fungal cell cycle. However, it would be correct to state that 14-3-3 binds/regulates different stages/cyclins etc. - but the actual role is much the

same. They state this in the introduction (bottom page 4 - 5). The fact that it plays a similar role as the Schizosaccharomcyes 14-3-3 would argue that the roles are conserved.

We agree the reviewer and changed accordingly this part of the text in the new version.

2. Materials and Methods: The plasmid section would read more smoothly if the primers were listed in a table (or supplemental data).

We included a Table with all primers.

3. Results. Top page 11. If Ustilago 14-3-3 plays a role in the cell cycle, how do the authors account for the observation that they did not see changes in cell distribution throughout the cell cycle when imaging the Bmh1-RFP strain?

We believe that the actual fraction of 14-3-3 protein involved in the interaction with Cdc25 is a low fraction of the total protein. In addition 14-3-3 has a pancellular distribution and probably it will be required an extremely sensitive microscopy system to detect such a subtle changes.

4. Fig. 2. E. Which are WT and which are mutant cells? All the cells in E look affected by the change in growth media.

In this new version we included a more clear figure.

5. Fig 3 B. What are the two bands in the whole cell extract?

The upper band is a non-specific cross-reacting band that is recognized on gel by the commercial anti-HA-HRP antibody. In some gels appears more than in others (compare WCE in figures 3B and 4D). However it is not immunoprecipitated from WCE when used the anti-HA antibody.

6. Fig 4 and Fig 5. The data is convincing that Cdc25p interacts with Ustilago Bmh1p and that the cdc25aaa mutant hinders interaction with Bmh1p . However, it is not clear that the actual interaction sites are mutated since 1) Co-IP still identifies interaction between Cdc25 aaa and Bmh1p. 2) The Cdc25aaa cells are viable. If Bmh1p sequesters Cdc25 in the nucleus then wouldn't this ultimately affect growth?

1) We mutated three putative sites that were sorted just by sequence analysis, but it is well known that 14-3-3 can bind to non-canonical sites. So we believe that our mutant may still bind 14-3-3 although with lower affinity and that explains the residual interaction. This has been also shown in the interaction between Cdc25C and 14-3-3 in Xenopus (Kumagai and Dunphy, 2007) and human cells (Giles et al., 2003). We included this explanation in the text.
2) We believe that there is still a residual ability to bind to 14-3-3, in such a way that even when affected cell cycle and morphology, the effects are less dramatic than the down-regulation of *bmh1*. Anyway, overexpression of Cdc25

affect cell cycle but is not lethal (Sgarlata and Perez-Martin, 2005). In addition we believe that the reason for the essentiality of 14-3-3 protein in *U. maydis* is not just its role controlling Cdc25, other putative targets, unknown so far, could contributed to the lethality effect.

7. Fig 4 A and B aren't really needed.

We believe that this figure is more clear with the schemes showed in 4A and 4B.

14-3-3 regulates the G2/M transition in the basidiomycete *Ustilago maydis*

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Running title: 14-3-3 and cell cycle regulation in smut fungus

Abstract

14-3-3 proteins are a family of highly conserved polypeptides that function as small adaptors that facilitate a diverse array of cellular processes by binding phosphorylated target proteins. One of these processes is the regulation of the cell cycle. Here we characterized the role of Bmh1, a 14-3-3 protein, in the cell cycle regulation of the fungus *Ustilago maydis*. We found that this protein is essential in *U. maydis* and that it has roles during the G2/M transition in this organism. The function of 14-3-3 in *U. maydis* seems to mirror the proposed role for this protein during *Schizosaccharomyces pombe* cell cycle regulation. We provided evidence that in *U. maydis* 14-3-3 protein binds to the mitotic regulator Cdc25. Comparison of the roles of 14-3-3 during cell cycle regulation in other fungal system let us to discuss the connections between morphogenesis, cell cycle regulation and the evolutionary role of 14-3-3 proteins in fungi.

1. Introduction

14-3-3 proteins represent a family of highly homologous proteins that have been described in all eukaryotic organisms from fungi to humans. These small acidic proteins have been implicated in a wide variety of cellular processes including signal transduction, cell cycle regulation and transcriptional regulation (Dougherty and Morrison, 2004). 14-3-3 monomers form cup-shaped homo- and hetero-dimers that are able to bind protein ligands, usually phosphorylated on serine or threonine residues of consensus binding motifs (Jones et al., 1995; Yaffe, 2002). There are several ways by which 14-3-3 proteins effect their function. These include altering the ability of the target protein to interact with other proteins, modifying the subcellular partitioning of a target, enhancing/inhibiting the catalytic activity of a target, protecting a target from dephosphorylation/proteolysis, and acting as a scaffold (Tzivion and Avruch, 2002).

Genes encoding 14-3-3 proteins have been described in different fungi. In *Saccharomyces cerevisiae* and *Scizosaccharomyces pombe* there are two 14-3-3 genes designated *BMH1* and *BMH2* (van Heusden et al., 1992; 1995) and *rad24* and *rad25* (Ford et al., 1992) respectively. In these fungi, deletion of one 14-3-3 gene has little effect but disruption of both renders the cell inviable in most strains. Supporting the essential role of 14-3-3 proteins in fungi, work in *Candida albicans* showed that in this fungus there is only one 14-3-3 protein-encoding gene and it is required for vegetative growth (Cognetti et al., 2002). In other fungi, like *Yarrowia lipolytica* (Hurtado and Rachubinski, 2002), *Aspergillus nidulans* (Kraus et al., 2002) and *Trichoderma reesei* (Vasara et al., 2002), genes encoding 14-3-3 proteins

have been reported although disruption of these genes in each organism has proven elusive.

The characterization of 14-3-3 homologues in fungi underpin the striking conclusion that in spite of their high conservation at sequence level (higher than 70% of amino acid identity), 14-3-3 proteins do not appear to be involved in the same processes in different fungi. An illustrative example is the different implication of 14-3-3 proteins during cell cycle regulation in *S. cerevisiae* and *S. pombe*. In *S. cerevisiae* 14-3-3 proteins seem to be required for G1/S transition, most likely at the level of the activity of the SBF (Swi4/6 Binding Factor) complex (Lottersberger et al., 2006). SBF is involved in the transcriptional activation of the *CLN1* and *CLN2* G1 cyclins, which are required for triggering both entry into S phase and cytoskeleton polarization for bud formation (Lew and Reed, 1993; Koch and Nasmyth, 1994). In contrast, 14-3-3 proteins in *S. pombe* are involved in the regulation of the G2/M transition through the negative regulation of the Cdc25 phosphatase (Lopez-Girona et al., 1999). In *S. pombe* the G2/M transition is controlled via the inhibitory phosphorylation of the cyclin-dependent kinase (CDK) by Wee1-related kinases (Lundgren et al., 1991). The Cdc25 phosphatase represents a key determinant of mitotic timing, as it is required to reverse the inhibitory phosphorylation of CDK thereby promoting entry into mitosis (Millar et al., 1991). Disruption of 14-3-3 encoding genes in *S. pombe* leads to premature entry into mitosis and it seems not to be involved in the regulation of the G1/S transition (Ford et al., 1992).

The observed differences between *S. cerevisiae* and *S. pombe* suggest that the function of 14-3-3 proteins during cell cycle co-evolved to support the

control of the most critical cell cycle transition in each organism. While in *S. cerevisiae*, the main cell cycle control is exerted during the G1/S transition, in *S. pombe* the G2/M transition is the key step during the cell cycle (Rupes, 2002). Hence, in *S. cerevisiae* G1 cyclins are essential and Mih1, the Cdc25-like phosphatase, is not required for viability. In contrast, in *S. pombe* G1 cyclins are dispensable while Cdc25 is essential (Rupes, 2002).

However, additional differences set apart both yeast, as *S. cerevisiae* divides by a budding process while *S. pombe* divides by fission. Since 14-3-3 proteins also play important roles in morphological level (van Heusden and Steensma, 2006), the different roles of 14-3-3 proteins in these two yeasts could also be related with the different morphological aspects.

We decided to study the role of 14-3-3 proteins during the cell cycle in the smut fungus *Ustilago maydis*. The reasoning behind the selection of *U. maydis* is because this phytopathogenic fungus offers a unique scenario to clarify the relative importance of cell cycle regulation versus morphology in the ascription of the role of 14-3-3 proteins. *U. maydis* cells normally produce one polar bud per cell cycle (Jacobs et al., 1994), as it happens in *S. cerevisiae*. However, like *S. pombe*, the G2/M transition is a main control and the Cdc25 phosphatase is essential while G1 cyclins are dispensable for growth (Castillo-Lluva and Pérez-Martín, 2005; Sgarlata and Pérez-Martín, 2005b; Pérez-Martín et al., 2006). In this report we provide evidence linking the role of 14-3-3 proteins in *U. maydis* to the control of the G2/M transition, supporting the interpretation that 14-3-3 protein roles co-evolved with the cell cycle regulation in the different fungi.

2. Materials and methods

2.1. Strains and growth conditions

Ustilago maydis strains are derived from FB1 background (Banuett and Herskowitz, 1989) and are listed in Table 1. Cells were grown in YPD (Sherman et al., 1986), complete medium (CM) or minimal medium (MM) (Holliday, 1974). Controlled expression of genes under the *crg1* and *nar1* promoters were performed as described previously (Brachmann et al., 2001; García-Muse et al., 2004). FACS analyses were described previously (García-Muse et al., 2003).

2.2. DNA, RNA, and protein analysis

U. maydis DNA isolation was performed as previously described (Tsukuda et al., 1988). RNA isolation and Northern analysis were performed as described previously (Garrido and Pérez-Martín, 2003). Protein extracts, immunoprecipitations and Western analysis, were performed as described previously (García-Muse et al., 2004; Sgarlata and Pérez-Martin, 2005b).

2.3. Plasmid and strain constructions

Plasmid pGEM-T easy (Promega) was used for cloning, subcloning and sequencing of genomic fragments and fragments generated by PCR (primers are listed in Table 2). Plasmids pRU11 and pRU2, to express genes under the control of P*crg1* and P*nar1* promoters respectively, were already described (Brachmann et al., 2001). Plasmid pBS-myc and pBS-ha, to produce C-terminal MYC- and HA-tagged protein fusions was already described (Garrido et al., 2004). Sequence analysis of fragments generated by PCR was

performed with an automated sequencer (ABI 373A) and standard bioinformatic tools. To construct the different strains, transformation of *U. maydis* protoplasts with the indicated constructions was performed as described previously (Tsukuda et al., 1988). Integration of the plasmids into the corresponding loci was verified in each case by diagnostic PCR and subsequent Southern blot analysis.

To produce a conditional *bmh1*^{crg1} allele, we constructed a plasmid by ligation of a pair of fragments into pRU11-T7, digested with *Nde*I and *Eco*RI. The 5' fragment (flanked by *Eco*RI and *Kpn*I) was produced by PCR using the primers 1433-9/1433-10 and spans from nucleotide -929 to nucleotide –200 (considering the adenine in the ATG as nucleotide +1). The 3' fragment (flanked by *Nde*I and *Kpn*I) was obtained by PCR amplification with primers 1433-7/1433-11 and spans from nucleotide +1 to nucleotide +1994. The resulting plasmid pBmh1crg1 was integrated, after digestion with *Kpn*I, by homologous recombination into the *bmh1* locus.

For C-terminal fusion of Bmh1 with RFP, and Cdc25 with GFP, the adaptation of the *Sfi*I-dependent gene replacement strategy for C-terminal tag described in Becht et al., (2006) was used. To produce Bmh1-RFP, two 5'- and 3'-fragments were digested with *Sfi*I and ligated to a cassette carrying the RFP-encoding gene as well as a nourseotricine resistance gene. The flanking 5' fragment was obtained after PCR amplification with primers 1433-13/1433-14 and spans from nucleotide +66 to nucleotide +1028. The flanking 3' fragment was obtained after PCR amplification with primers 1433-15/1433-16 and spans from nucleotide +1033 to nucleotide +2052. To produce Cdc25-3GFP, two 5'- and 3'- fragments were digested with *Sfi*I and ligated to a cassette

carrying a triple GFP-encoding gene as well as a hygromycin resistance gene. The flanking 5' fragment was obtained after PCR amplification with primers cdc25-gfp2/cdc25-gfp3 and spans from nucleotide +1974 to nucleotide +2969. The flanking 3' fragment was obtained after PCR amplification with primers cdc25-gfp4/cdc25-gfp5 and spans from nucleotide +3007 to nucleotide +4009. The C-terminal myc-tagged Cdc25 proteins was already described (Sgarlata and Pérez-Martín, 2005b). A similar procedure was used to tag $Cdc25^{AAA}$.

To tag Bmh1 with a 3HA epitope, a kb, a *Nde*I-*Eco*RI carrying the entire *bmh1* ORF sequence without the stop codon was obtained by PCR amplification of *U. maydis* genomic DNA with the primers 1433-7/1433-19 and this fragment was cloned into the plasmid pBS-ha. This plasmid was linearized with *Stu*I and integrated into the *bmh1* locus by homologous recombination.

To construct the $cdc25^{AAA}$ mutant allele, a two-step mutagenesis protocol using PCR. Ser214, Ser 460, and Thr584 residues were exchanged with Ala residues. Each change created a new restriction site (*Pfl*MI, *Pst*I y *Xma*I, respectively) that was used to track the mutation by PCR amplification of genomic DNA. The first round of mutagenesis (Ser214 to Ala 214) was performed with the primers cdc25-27/cdc28-28. The second mutagenesis round (exchanging Ser460 with Ala460) was performed with primers cdc25-29/cdc25-30. Finally the third mutation (Ala584) was introduced with the pair of primers cdc25-31/cdc25-32. The mutated allele was introduced into the corresponding locus by homologous integration.

2.4. Microscopic observations

Samples were mounted on microscope slides and visualized in a Nikon eclipse 90i microscope equipped with a Hamamatsu ORCA-ER CCD camera. Images were taken using the appropriate filter sets, a Nikon Plan Apo VC x100 NA 1.40 lens and Nikon Immersion oil Type A nd=1.515. The software used with the microscope was MetaMorph 6.1 (Universal Imaging, Downingtown, PA). Images were further processed with Adobe Photoshop 7.0. All the images in this study are single planes. Standard FITC and DAPI filter sets were used for epifluorescence analysis of nuclear staining with DAPI (García-Muse et al., 2003) and WGA staining, performed as described (Castillo-Lluva et al., 2004). The ratio of the nuclear intensity (N) to the cytoplasmic intensity (C) was calculated as described in (Dunaway et al., 2005). Briefly, the intensity of the nuclear and cytoplasmic signal was determined by measuring pixel intensity in the nucleus and of an equivalent area in the cytoplasm and the ratio was determined. 60 cells were quantified for each experiment.

Sequence analyses. Protein sequences of fungal 14-3-3 proteins were downloaded from PubMed (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi). Alignments and phylogenetic dendrograms were constructed using ClustalW and NJPlot programs (Thompson et al., 1997). Bmh1 was identified in the genomic sequence of *U. maydis* (http://www.broad.mit.edu/annotation/fungi/ustilago_maydis/index.html) as a hypothetical protein UM01366.

3. Results

3.1. U. maydis genome encodes a single 14-3-3 protein that is essential

We used the sequences of the described *S. cerevisiae*, *S. pombe* and *C. albicans* 14-3-3 proteins to carry out a BLAST search against *U. maydis* genomic sequence data. We found a single hit, corresponding to the predicted protein UM01366 in the annotated *U. maydis* genome database (http://mips.gsf.de/genre/proj/ustilago/). We named this protein Bmh1 by analogy to *S. cerevisiae*. Comparison of *U. maydis* Bmh1 with the fungal homologues indicated a level of sequence identity ranging from 70 to 79%. This high identity is located at the central core were the α-helices responsible for the cup-shape structure of the protein are located. In contrast, this group of proteins shares little sequence similarity within the carboxy-terminal tail region (Fig. 1A).

Although 14-3-3 homologs can be found in almost all fungal genomes sequenced, only a very few of them have been characterized. A dendrogram analysis of the predicted amino acid sequence of *U. maydis* Bmh1 and the rest of fungal 14-3-3 proteins characterized so far is shown in Fig. 1B. Most fungi have more than one gene encoding 14-3-3 proteins, but there are cases like *C. albicans* where only one single gene is present. In this case, the 14-3-3–encoding gene has been proved to be essential (Cognetti et al., 2002). As our attempts to delete the *bmh1* gene in haploid *U. maydis* cells were elusive, we inactivated one *bmh1* allele in the diploid FBD11 strain, replacing it with a hygromycin resistance cassette, generating the Δ*bmh1* null allele. When the meiotic progeny of this strain were analyzed after sporulation (Holliday, 1974),

no hygromycin resistant haploid cells were found (not shown) indicating that *bmh1* is an essential gene.

14-3-3 proteins regulate proteins located both at the cell nucleus and the cytoplasm. In order to analyze the subcellular location of Bmh1 in *U. maydis*, we fused a RFP-tag to the C-terminus of the endogenous *bmh1* gene in wild-type cells. This fusion protein was functional, as the strain carrying the tagged version of Bmh1 was indistinguishable from wild-type cells. We detected a clear Bmh1-RFP signal in both the cytoplasm and the nucleus with a slightly higher concentration of Bmh1-RFP in the nucleus (Fig. 1C). We did not observe changes in this distribution depending of the cell cycle stage of the cell (unpublished observations).

3.2. Down-regulation of bmh1 strongly affects the cell morphology

To further analyze the function of Bmh1 during cell cycle in *U. maydis*, we constructed a strain in which the Bmh1 protein could be conditionally depleted. This was achieved using the *U. maydis crg1* promoter that is induced by growing the cells in arabinose as the carbon source, and strongly repressed in glucose-containing medium (Bottin et al., 1996). A chimeric allele (*bmh1*^{crg1}) was constructed by fusing the *crg1* promoter to the coding region of *bmh1* and the native allele was replaced by this conditional allele (Fig. 2A). In the conditional mutant, a strong decrease in the levels of *bmh1* mRNA was observed when cells were incubated in restrictive conditions (YPD, Fig. 2B). In contrast, the *bmh1* mRNA levels were around 5-fold higher than wild-type control at permissive conditions (YPA, Fig. 2B). In accordance with the

essential role of Bmh1, conditional cells were unable to form colonies when shifted to glucose-containing solid medium (Fig. 2C).

We analyzed the growth of this conditional strain in liquid medium. In permissive conditions, conditional cells were indistinguishable from wild-type cells in morphology (Fig. 2D) and growth rate (not shown). However, at restrictive conditions the conditional cells generated less elongated cells that frequently divided by septation (Fig. 2E). Prolonged incubation (more than 12 hours) at restrictive conditions produced irregular shaped cell aggregates where most cells did not separate, some cells lost their polarity and became rounded while others remained elongated (Fig. 2F). In average, the cell compartments were shorter than normal (10±3 μm versus 17±2 μm in wild-type *U. maydis* cells, n=50 cells or cell compartments) and contained at least one nucleus (Fig. 2E, F).

3.3. Bmh1 affects the subcellular localization of Cdc25

The cell aggregates observed when *bmh1* expression was down-regulated were reminiscent of those seen in cells overexpressing the phosphatase *cdc25* or when the Wee1 protein kinase was conditionally removed (Sgarlata and Pérez-Martín, 2005a, b). In both cases, the levels of inhibitory phosphorylation of Cdk1 -the catalytic subunit of the mitotic cyclin-dependent kinase- were disminished and as a consequence cells enter prematurely into mitosis and were unable to produce a bud, therefore leading to division through septation (Pérez-Martín et al., 2006). To address whether down-regulation of *bmh1* in *U. maydis* may affect the level of Cdk1 inhibitory phosphorylation, we examined the phosphotyrosine content of Cdk1 in

conditional cells growing in restrictive conditions for *bmh1* expression (glucose-containing medium). For this, we used a specific antibody raised against the phosphorylated human Cdc2-$Y^{15}P$ peptide, which recognize the Tyr15-phosphorylated form of *U. maydis* Cdk1 (Sgarlata and Pérez-Martín, 2005a). We found a decrease of around 10-fold in the levels of $Tyr^{15}P$-Cdk1 when *bmh1* expression was repressed in the conditional cells (Fig. 3A).

In line with the described negative regulation of the mitotic promoter Cdc25 by 14-3-3 proteins in *S. pombe* (Ford et al., 1992), our interpretation of these results was that in *U. maydis* Bmh1 could also act as a repressor of the Cdc25 phosphatase. To gain further support in this hypothesis, we analyzed whether Cdc25 is able to interact *in vivo* with 14-3-3. For this we constructed a strain carrying a HA tagged version of Bmh1 and a 3MYC-tagged C-terminal version of Cdc25. Both epitope-tagged proteins were as functional as their respective wild-type alleles (not shown, Sgarlata and Pérez-Martín, 2005b). Cell lysates were immunoprecipitated with anti-MYC and anti-HA antibodies, and the immunoprecipitates were analyzed for Cdc25 (using anti-MYC antibodies) and Bmh1 (using anti-HA antibodies). We found that 14-3-3 protein and Cdc25 co-immunoprecipitated (Fig. 3B), supporting the conclusion that both proteins were able to physically interact.

In human, *Xenopus laevis* and *S. pombe* cells, Cdc25 proteins are kept inactive by cytoplasmic sequestration mediated by the association to 14-3-3 proteins during normal cell cycle (Kumagai and Dunphy, 1999; Lopez-Girona et al., 1999; Graves et al., 2001). Therefore we analyzed the subcellular localization of Cdc25 using a C-terminal 3GFP-tagged functional protein. The Cdc25-GFP signal was observed both in cytoplasm and nucleus, although a

weak nuclear accumulation was apparent (Fig. 3C, left panels). However, we observed that the Cdc25-GFP nuclear signal was more intense in *bmh1*^{crg1} conditional cells when incubated in restrictive conditions for *bmh1* expression (Fig. 3C, right panel).

These results were consistent with the idea that in *U. maydis* the 14-3-3 protein Bmh1 could be involved in the control of the mitotic inducer Cdc25.

3.4. A Cdc25 mutant protein impaired in the ability to bind 14-3-3 proteins showed a shorter G2 phase

To further support the hypothesis that 14-3-3 proteins control Cdc25 in *U. maydis*, we sought to analyze the effect in the cell of a Cdc25 mutant version impaired in the ability to bind to Bmh1. Work performed in *S. pombe*, human and *X. laevis* indicated that the interaction between Cdc25 and 14-3-3 proteins was mediated by the recognition of specific binding sites, which overlap with the target sites for a family of kinases that include, among others, the DNA damage checkpoint kinase Chk1 (Kumagai et al., 1998; Zeng et al., 1998; Kumagai and Dunphy, 1999; Forrest and Gabrielli, 2001; Gilles et al., 2003). In these binding sites, the phosphorylation of the serine or threonine by the respective kinase is crucial for the interaction with 14-3-3 proteins. Exchange of the serine or threonine residues with alanine reduces the binding of 14-3-3 proteins to Cdc25. The alignment of the different 14-3-3 recognition sequences present in Cdc25 from human, *X. laevis* and fission yeast led to a consensus sequence, ϕxRxx[S/T]xϕ, where ϕ indicates hydrophobic residues and the Ser/Thr residue is the target of kinases from the Chk1 family (Fig. 4A).

We analyzed the amino acid sequence of *U. maydis* Cdc25 using the ELM algorithm (http://elm.eu.org/links.html) and found that five putative binding sites for 14-3-3 proteins were predicted. However, after sorting these *U. maydis* sequences with the above proposed consensus sequence for the 14-3-3 recognition sites in Cdc25, only two putative binding sites (Ser460 and Thr584) fulfilled such criteria. A third one (Ser214), which had a conservative histidine to arginine substitution at the position –3, was also selected since it was located nearby the NLS, a critical place as it has been shown in mammalian Cdc25B and Cdc25C (Forrest and Gabrielli, 2001; Graves et al., 2001). Therefore, we decided to exchange these serine and threonine residues to alanine to generate the triple mutant allele, *cdc25*^{AAA} (Figs. 4A, B)
To analyze the behavior of cells carrying the triple mutant allele, *cdc25*^{AAA}, we firstly demonstrated that this mutant allele was functional by introducing the *cdc25*^{AAA} allele into a conditional strain carrying the *cdc25*^{nar1} allele (Sgarlata and Pérez-Martín, 2005b). At restrictive conditions, the *cdc25*^{AAA} allele rescued the cell cycle arrest of *cdc25*^{nar1} cells, indicating that it was functional (Fig. 4C). Secondly, we tested whether the ability of Cdc25 to interact *in vivo* with 14-3-3 was affected in the triple mutant with respect the wild-type. For this, we introduce a 3myc-tagged C-terminal version of Cdc25^{AAA} in a strain already carrying a HA tag in Bmh1. Lysates prepared from the corresponding cells were immunoprecipitated with anti-MYC and anti-HA antibodies, and the immunoprecipitates were analyzed for Cdc25 (using anti-MYC antibodies) and Bmh1 (using anti-HA antibodies). In agreement with our proposal, we found that the triple mutant displayed severely reduced 14-3-3 binding compared with wild-type protein (Fig.4D). This strongly suggests that the proposed sites

are *in vivo* 14-3-3 binding sites. However, it is worth to keep on mind that there is still a residual binding activity, most likely because the ability of Bmh1 to bind to non-canonical sites present in Cdc25. These secondary sites have been described in Xenopus and human Cdc25C proteins (Kumagai and Dunphy, 1999; Gilles et al., 2003).

We observed that the cells carrying the *cdc25*^{AAA} allele were shorter in size in comparison to control cells (Fig. 5A). As this could be a consequence of entering mitosis prematurely, we decided to analyze the DNA content of control and mutant cells growing in different medium, as the proportion between G1 and G2 length is adjusted depending on nutritional conditions (Garrido and Pérez-Martín, 2003; Castillo-Lluva et al., 2004). We observed that the proportion of cells with a 2C DNA content (i. e. G2 and M phase cells) was lower in the mutant strain in comparison to the control strain, independently of the culture medium tested. In contrast, there is a clear increase in cells with 1C DNA content (i. e. G1 cells) in comparison to control cells growing in the same conditions (Fig. 5B). *U. maydis*, as many other types of cells compensate for induced alteration in the length of one cell cycle phase (G1, S or G2) by altering the length of the other phases (Rupes, 2002; Pérez-Martín et al., 2006). We believe that the bias towards cells with 1C DNA content in the *cdc25*^{AAA} cells was the result of a compensatory mechanism by which the G1 period is lengthened in response to a shorter G2 phase.

We also analyzed the level of Cdk1 tyrosine phosphorylation in cells carrying the *cdc25*^{AAA} allele in comparison to wild-type cells. Consistently, we found lower levels of Tyr15 phsophorylation in mutant cells, (Fig. 5C) supporting the

idea that removal of 14-3-3 binding sites resulted in higher levels of *in vivo* Cdc25 activity.

Given the observations that Cdc25 interacts with 14-3-3 proteins and that it seems to affect the subcellular localization of Cdc25, we investigated the localization of $Cdc25^{AAA}$. For this we tagged the *cdc25*^{AAA} allele with a GFP fusion and analyzed the nucleus/cytoplasm distribution of the fusion protein. To quantitatively evaluate changes in localization, fluorescence intensities of the Cdc25 signal were determined for a circular region corresponding to the nucleus (N) and a comparable area in the cytoplasm (C), as explained in the Materials and Methods. We found that the ratio N/C was higher in the mutant allele than in the wild-type (Fig. 5D), supporting a direct role of 14-3-3 in cytoplasmic retention of Cdc25 in *U. maydis*.

Altogether these data suggest that control of Cdc25 by 14-3-3 is required for appropriated G2 phase length in *U.maydis.*

3.5. Bmh1 is required for the DNA damage response

14-3-3 proteins have been implicated in DNA damage-dependent cell cycle regulation in *S. cerevisiae* and *S. pombe* (Ford et al., 1992; Chan et al., 1999; Lopez-Gironal et al., 1999; Lottersberger et al., 2003). Therefore we analyzed the sensitivity of *U. maydis* cells defective in 14-3-3 proteins to UV radiation. As *bmh1* was an essential gene, conditional *bmh1*^{crg1} and control cells were grown in permissive conditions for *bmh1*^{crg1} expression (arabinose-containing medium) and then transferred to restrictive conditions (glucose-containing medium) for several hours in order to deplete the intracellular pool of Bmh1. Afterwards, ten-fold dilutions of these cells were plated in arabinose-

containing solid medium. These plates were immediately irradiated with different doses of UV light (150 and 300 J/m^2), incubated for several days, and the proportion of survival cells counted (Fig. 6A). We found that in conditions in which Bmh1 protein was absent at the time of UV exposition, cells were around 100-fold more sensitive than wild-type to UV radiation (Fig. 6C).

These results support the idea that, as it happens in *S. cerevisiae* and *S. pombe*, *U. maydis* 14-3-3 protein is involved in the response to DNA damage. In *S. cerevisiae* the 14-3-3 checkpoint functions are related with the ability to activate the checkpoint kinase Rad53, but has no effect in the control of the activity of Mih1, the Cdc25-like phosphatase (Usui and Petrini, 2007). In contrast, in *S. pombe* 14-3-3 proteins have at least two roles: to activate the checkpoint kinase Chk1 (Dunaway et al., 2005), and to target the Cdc25 phosphatase (Lopez-Girona et al., 1999). To discriminate whether the roles of *U. maydis* 14-3-3 proteins during the DNA damage checkpoint are related to the ability to down-regulate Cdc25, we analyzed the UV sensitivity of cells carrying the *cdc25^{AAA}* allele (Fig. 6B). Our results, indicating around a 10-fold more sensitivity in the *cdc25^{AAA}* cells than control cells (Fig. 6C), support the idea that the mechanism behind the checkpoint response in *U. maydis* could be more related to the one observed in *S.pombe*.

4. Discussion

The budding yeast *S. cerevisiae* and the fission yeast *S. pombe* have been excellent genetic models to study cell cycle control in fungi for long time. Both yeasts share cell cycle characteristics, similar CDK regulatory systems, and

checkpoint controls. However, below these general principles and common elements, each system has particularities. In these yeasts, it has been proved that cell cycle control is intricately linked to morphogenesis (Lew and Reed, 1993; Jorgensen and Tyers, 2004; McCusker et al., 2007). Hence, the distinct growth mode and division of both organisms preclude different ways to regulate the two main cell cycle transitions, G1/S and G2/M. In *S. cerevisiae* cells, the G1/S transition is where the main controls are applied while in *S. pombe* is the G2/M transition (Rupes, 2002). *U. maydis* is a budding yeast and therefore it was plausible to assume that cell cycle regulation will follow similar principles as those described in *S. cerevisiae*. However, the previous characterization of cell cycle regulators in *U. maydis* suggested that in this fungus the regulation of cell cycle was more similar to *S. pombe* than to *S. cerevisiae* (Pérez-Martín et al., 2006). For instance, inhibitory phosphorylation of Cdk1, crucial for fission yeast but dispensable for *S. cerevisiae*, is essential in *U. maydis* (Sgarlata and Pérez-Martín, 2005a). And so is true for cell cycle regulators related with this task (i. e the kinase Wee1 and the phosphatase Cdc25; Sgarlata and Pérez-Martín, 2005a, b).

In this report, we have continued our approach to define key cell cycle regulators in *U. maydis* and we have identified and characterized the 14-3-3 proteins. We showed that *U. maydis* contains a single class of 14-3-3 proteins, encoded by the gene *bmh1*. Although 14-3-3 homologs can be found in almost all fungal genomes sequenced, only a very few of them have been characterized. Most fungi have more than one gene encoding 14-3-3 proteins, but there are cases like *C. albicans* where only one single gene is present (Cognetti et al., 2002). Our functional analysis point towards the conclusion

that the role of 14-3-3 protein during cell cycle in *U. maydis* mirrors the proposed role of 14-3-3 proteins in *S. pombe* cell cycle: to negatively control the phosphatase Cdc25 and therefore to act as a brake for unscheduled mitosis. Several data support this conclusion: i) Bmh1 and Cdc25 physically interact, and this interaction can be impaired by several mutations in Cdc25 altering the putative 14-3-3 binding sites; ii) Decrease of Bmh1 levels or mutations in Cdc25 that affect the ability to bind to Bmh1 resulted in an decrease in the levels of inhibitory phosphorylation of Cdk1; iii) Affecting the ability of Cdc25 to bind Bmh1 or decreasing the levels of Bmh1 alters the subcellular localization of Cdc25; iv) Cdc25 mutants impaired in the binding to Bmh1 showed a shorter G2 phase.

We believe that, alike *S. pombe*, this scheme of regulation can be also applied to the DNA damage response, where 14-3-3 may be acting through Cdc25, among other effectors such as a putative Chk1-like kinase.

In conclusion, our findings reinforce the impression that cell cycle regulation in *U. maydis* follows a scheme comparable to *S. pombe*, in spite of the fact that *U. maydis* is a budding yeast. Then, it is fair to conclude from these results that morphogenesis and cell cycle evolved in an independent way in each fungal system. However, we believe that this is a naive conclusion that deserves some clarifications. Similar to *S. cerevisiae*, vegetative growing *U. maydis* cells normally produce one polar bud per cell cycle (Jacobs et al., 1994). However, two main differences distinguish bud formation in both yeasts. Firstly, studies correlating nuclear density and cell morphology showed that *U. maydis* cells complete DNA synthesis before beginning to form buds (Snetselaar and McCann, 1997). In other words, the bud formation

takes place in G2 phase, in contrast to bud formation in *S. cerevisiae* that is reported to occur during S phase (Pringle and hartwell, 1981). The second difference relates to the amount of polar growth required for bud formation. *S. cerevisiae* cells undergo a brief period of polar growth during bud emergence, but then switch to isotropic growth that occurs over the entire surface of the bud (Lew and reed, 1993). In *U. maydis*, in contrast, the growth of the bud relies almost entirely on polar growth (Steinberg et al., 2001). In fact, the growth mode of *U. maydis* is reminiscent of *S. pombe,* where all growth occurs at the end of the cells (polar growth) and the activation of the growth at the new end (New End Take Off, NETO) occurs once DNA has been replicated (Mitchison and Nurse, 1985). Therefore, we want to propose that the similarities between the cell cycle regulation in *S. pombe* and *U. maydis* are a consequence of the strong requirement in polar growth rather than other morphological features such as division by fission or budding. These connections between polar growth and cell cycle regulation were already noted by Kellog (2003) to explain the different requirement between *S. pombe* and *S. cerevisiae* with respect the inhibitory phosphorylation.

14-3-3 proteins are rather flexible control elements, acting in different system and in distinct ways, just in virtue of its ability to regulate proteins simply by binding to them. Thereby, they are able to act as a wildcard to enhance the regulatory control of almost any process. Accordingly, the number of proteins potentially regulated by the association to 14-3-3 proteins may be in the range of several hundreds (Jin et al., 2004; Meek et al., 2004; Pozuelo-Rubio et al., 2004). We believe that in fungal cells, during the process of co-evolution between cell cycle regulation and morphogenesis, for each organism, 14-3-3

proteins were recruited to reinforce the control during the more crucial step of the cell cycle. In this way, in spite of the sequence similarity observed among the different 14-3-3 proteins in fungi, their role reflects the particularities of each cell cycle/morphogenesis regulation.

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Legends to Figures

Fig. 1. 14-3-3 protein in *U. maydis*. (A) A multiple sequence alignment of fungal 14-3-3 proteins. Lines on sequences blocks indicate the position of putative alpha helices in the three-dimensional structure. Identical amino acids are indicated in black boxes while conservative changes are indicated in gray boxes. (B) Dendrogram of fungal 14-3-3 proteins. The tree was reconstructed using the ClustalW method (http://www.ebi.ac.uk/clustalw/). Bar=0,05 substitutions per aa. Numbers in the nodes indicate branch distances. The proteins utilized were *Schizosaccharomyces pombe* Rad24 (SpRad24; CAA55795) and Rad25 (SpRad25; CAA55796); *Saccharomyces cerevisiae* Bmh1 (ScBmh1; CAA46959) and Bmh2 (ScBmh2; CAA59275); *Candida albicans* Bmh1 (CaBmh1; AAB96910); *Aspergillus nidulans* ArtA (AnArtA; AAK25817) and the putative protein An5744 (An5744.2; XP66348.1); *Yarrowia lipolytica* Bmh1 (YlBmh1; AAM09811) and Bmh2 (YlBmh1; AAM09812); *Trichoderma reesei* TrFtt1 (CAC20377) and TrFtt2 (CAC20378); and *Ustilago maydis* Bmh1 (UmBmh1; XP757513.1). (C) Subcellular distribution of a Bmh1-RFP fusion. UMP120 cells were grown in CMD medium until exponential phase. Bar: 10 μm.

Fig. 2. Conditional removal of Bmh1. (A) Scheme of the conditional allele *bmh1*^{crg1}. (B) Levels of *bmh1* mRNA in the conditional strain are shown. The wild type FB1 and conditional MUM65 (*bmh1*^{crg1}) strain were grown for 8 hours in permissive (YEP medium plus arabinose as carbon source, YPA) or restrictive conditions (YEP medium plus glucose as carbon source, YPD). The RNA was extracted and analyzed by Northern blotting, loading 10 μg total

RNA per lane. *18s* rRNA was used to control for loading. (C) Growth of conditional strain in solid medium. Serial tenfold dilutions of FB1 (WT) and MUM65 (*bmh1*^{crg1}) cultures were spotted in solid YPD and YPA. Plates were incubated for 2 days at 28ºC. (D) Morphology of wild type and mutant cells incubated for 8 hours in permissive conditions (YPA). Cells were stained with DAPI to visualize their nuclei and FITC-conjugated Wheat Germ Agglutinin (WGA) to stain chitin. Bar=10 μm. (E) Morphology of wild type and mutant cells incubated for 8 hours in restrictive conditions (YPD). Cells were stained with DAPI. Bar=6 μm. (F) Morphology of mutant cells incubated for 14 hours in restrictive conditions (YPD). Cells were stained with DAPI to visualize their nuclei and FITC-conjugated Wheat Germ Agglutinin (WGA) to stain chitin. Bar=6 μm.

Fig. 3. Bmh1 interacts with the phosphatase Cdc25. (A) Western analysis of inhibitory phosphorylation of Cdk1 after *bmh1* down-regulation. Protein extracts from FB1 (wt) and MUM65 (*bmh1*^{crg1}) cultures incubated in repressing conditions for *crg1* expression (YPD) for the times indicated were obtained. The *bmh1*^{crg1} allele has a T7 N-terminal tag that allows the detection using anti-T7 antibodies. Cdk1 was visualized with anti-phospho-Cdc2 (Tyr15) and anti-PSTAIRE antibodies. Anti-PSTAIRE antibodies detect both Cdk1 and Cdk5 (Castillo-Lluva et al., 2007). (B) Physical interaction between Cdc25 and 14-3-3 protein in *U. maydis.* UMP125 cells (Bmh1-3HA Cdc25-3MYC) were grown for 8 hours in CMD and then harvested. Whole cell extracts (WCE) of these cells were independently immunoprecipitated with anti-MYC and anti-HA, and lysates (as input control) and immunoprecipitates were

immunoblotted for Myc-tagged Cdc25 and HA-tagged Bmh1. (C) Localization of Cdc25-3GFP in wild-type and *bmh1*^{crg1} cells. MUM72 cells carrying a endogenous functional Cdc25-3GFP as well as MUM81 cells carrying the conditional allele *bmh1*^{crg1} and the Cdc25-3GFP fusion were grown in CMD (restrictive condition for *bmh1*^{crg1}) and CMA (permissive condition for *bmh1*^{crg1}) for 6 hours before microscopy observation. Bar=20 μm.

Fig. 4. Identification of the 14-3-3 sites in Cdc25. (A) Comparison of the different described 14-3-3 binding sites in Cdc25. Below is shown the consensus 14-3-3 binding site, depicting the phosphorylated Ser/Thr residue. ϕ indicated an hydrophobic amino acid. On the right the sequence of the three potential sites in the *U. maydis* Cdc25 protein. (B) Schematic representation of Cdc25 sequence showing the relative positions of the putative 14-3-3 binding sites with respect to the phosphatase catalytic domain (PPase) as well as the putative NLS. (C) Cdc25^{AAA} is a functional protein. Serial tenfold dilutions of FB1 (wt), UMC27 (*cdc25*^{nar1}), MUM67 (*cdc25*^{nar1}/*cdc25*) and MUM66 (*cdc25nar1*/*cdc25*^{AAA}) cultures were spotted in solid rich medium (YPD) and minimal medium with nitrate (MMNO_3). YPD plates were incubated for 2 days and the nitrate plates for 4 days at 28ºC. (D) Physical interaction between Cdc25 and 14-3-3 protein in *U. maydis.* UMP125 cells (Bmh1-3HA Cdc25-3MYC) and UMP126 cells (Bmh1-3HA Cdc25^{AAA}-MYC) were grown for 8 hours in CMD and then harvested. Whole cell extracts (WCE) of these cells were independently immunoprecipitated with anti-MYC and anti-HA, and lysates (as input control) and immunoprecipitates were immunoblotted for Myc-tagged Cdc25 and HA-tagged Bmh1.

Fig. 5. Phenotypic characterization of cells carrying the *cdc25*^{AAA} allele. **(**A) Morphology of control (MUM58) and mutant (MUM57) cells incubated for 8 hours in CMD. Cells were stained with DAPI to visualize their nuclei. Note that mutant cells are shorter in length. Bar=15 μm. (B) DNA content of control (MUM58) and mutant (MUM57) cells growing in different media. (C) Levels of inhibitory Cdk1 phosphorylation in *cdc25*^{AAA} cells. Protein extracts from MUM58 (control) and MUM57 (*cdc25*^{AAA}) cultures were used for Western blot analysis. Cdk1 was visualized with anti-phospho-Cdc2 (Tyr15) and anti-PSTAIRE antibodies. (D) Quantification of nucleus/cytoplasm ratio of Cdc25-GFP immunofluorescence signal. Quantification of immunofluorescence signal was performed by measuring pixel intensity in the nuclei and in an equivalent area of the cytoplasm. Cells were grown in CMD for 6 hors before microscopy observation.

Fig. 6. Bmh1 is required for the DNA damage response. (A) UV sensitivity of *bmh1*^{crg1} cells. Wild-type and *bmh1*^{crg1} cells were grown for 10 hours in restrictive conditions for *crg1* expression (YPD) to deplete the internal pool of Bmh1 and then serial tenfold dilutions of were spotted in solid rich medium with arabinose (YPA) and immediately exposed to different UV intensities. Plates were incubated for 2 days at 28ºC. (B) Serial tenfold dilutions of control and *cdc25*^{AAA} cultures were spotted in solid rich medium (YPD) and immediately exposed to different UV intensities. Plates were incubated for 2 days at 28ºC. (C) Survival of cells after irradiation with the indicated doses of UV.

Table 1. *U. maydis* strains used in this study

Strain	Relevant genotype	Reference
FB1	*a1 b1*	Banuett and Herskowitz, 1989
UMC27	*a1 b1 cdc25^{nar1}*	Sgarlata and Pérez-Martín, 2005b
UMP120	*a1 b1 bmh1-RFP*	This work
UMP125	*a1 b1 bmh1-3HA cdc25-3myc*	This work
UMP126	*a1 b1 bmh1-3HA cdc25^{AAA}-3myc*	This work
UMP130	*a1 b1 cdc25^{AAA}-3GFP*	This work
MUM57	*a1 b1 cdc25^{AAA}-3myc*	This work
MUM58	*a1 b1 cdc25-3myc*	This work
MUM65	*a1 b1 bmh1^{crg1}*	This work
MUM66	*a1 b1 cdc25^{nar1} P$_{cdc25}$- cdc25^{AAA}*	This work
MUM67	*a1 b1 cdc25^{nar1} P$_{cdc25}$-cdc25*	This work
MUM72	*a1 b1 cdc25-3GFP*	This work
MUM81	*a1 b1 bmh1^{crg1} cdc25-3GFP*	This work

Table 2. Oligonucleotides used in this study

name	Sequence
1433-7	5'CATATGCCCGAATCGCGTGAAGACTCTGTC3'
1433-9	5'GAATTCTTGCTACTGGATCTGCGACAAAGC3'
1433-10	5'GGTACCCTCATTGTTATCGCTTCACTCTCC3'
1433-11	5'GGTACCAGCGACTAGCGTCAAACGAACAGC3'
1433-13	5'TTAATTAAGTAGCAGCGACAAAAACCTGAC3'
1433-14	5'TCTGGCCGCGTTGGCCGCGGCCTCCTCACCCTTGGCCTCGGCAG3'
1433-15	5'ATAGGCCTGAGTGGCCGACCTCTCCTTCTTGCTCCCCTCCCC3'
1433-16	5'TTAATTAACACTCACGACTCACGACTGCAC3'
1433-19	5'GACGAATTCGGCGGCCTCCTCACCCTTGGCCTC3'
cdc25-gfp2	5'TTAATTAACAACGTCAAGGATGACGGACTC3'
cdc25-gfp3	5'GGTGGCCGCGTTGGCCGACATATGTCGCGACAAGATGTT3'
cdc25-gfp4	5'ATAGGCCTGAGTGGCCAGCAGCATGTCGCAGCCGACGAC3'
cdc25-gfp5	5'TTAATTAACTGGAACGGGTATGCTCTGCGC3'
cdc25-27	5'CTGGCCCACGCCATGGCTTCGCGCCACTTTGGC3'
cdc25-28	5'GCCAAAGTGGCGCGAAGCCATGGCGTGGGCCAG3'
cdc25-29	5'GTGCCCAGGGCAGCTGCAGCCTCCAGCTCAAGC3'
cdc25-	5'GCTTGAGCTGGAGGCTGCAGCTGCCCTGGGCAC3'

30	
cdc25-31	5’CCTGCGATGCGAGCCCGGGCGCGCCCCCATTATTTACGC3’
cdc25-32	5’GCGTAAATAATGGGGGCGCGCCCGGGCTCGCATCGCAGG3’

Figure

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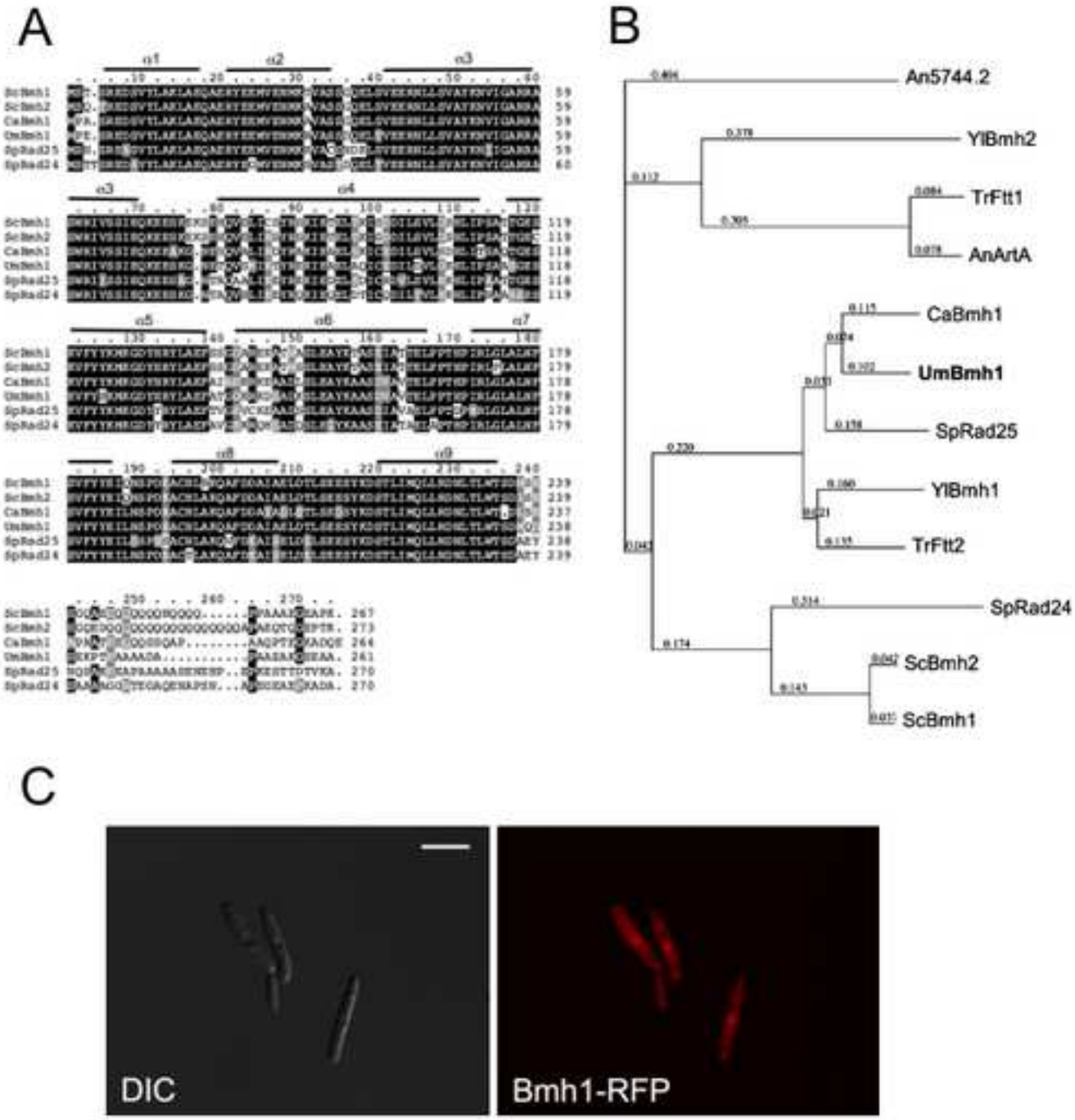

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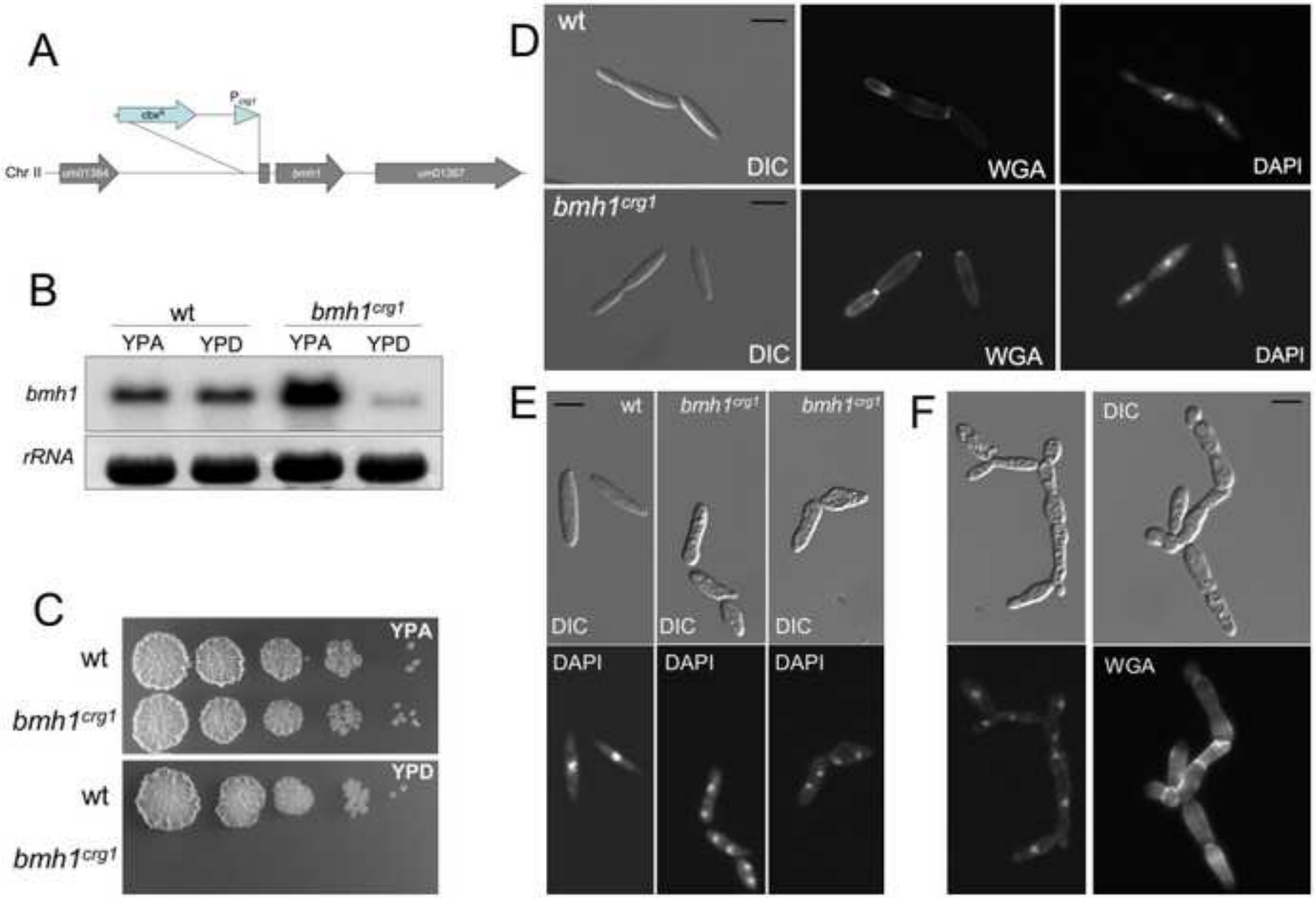

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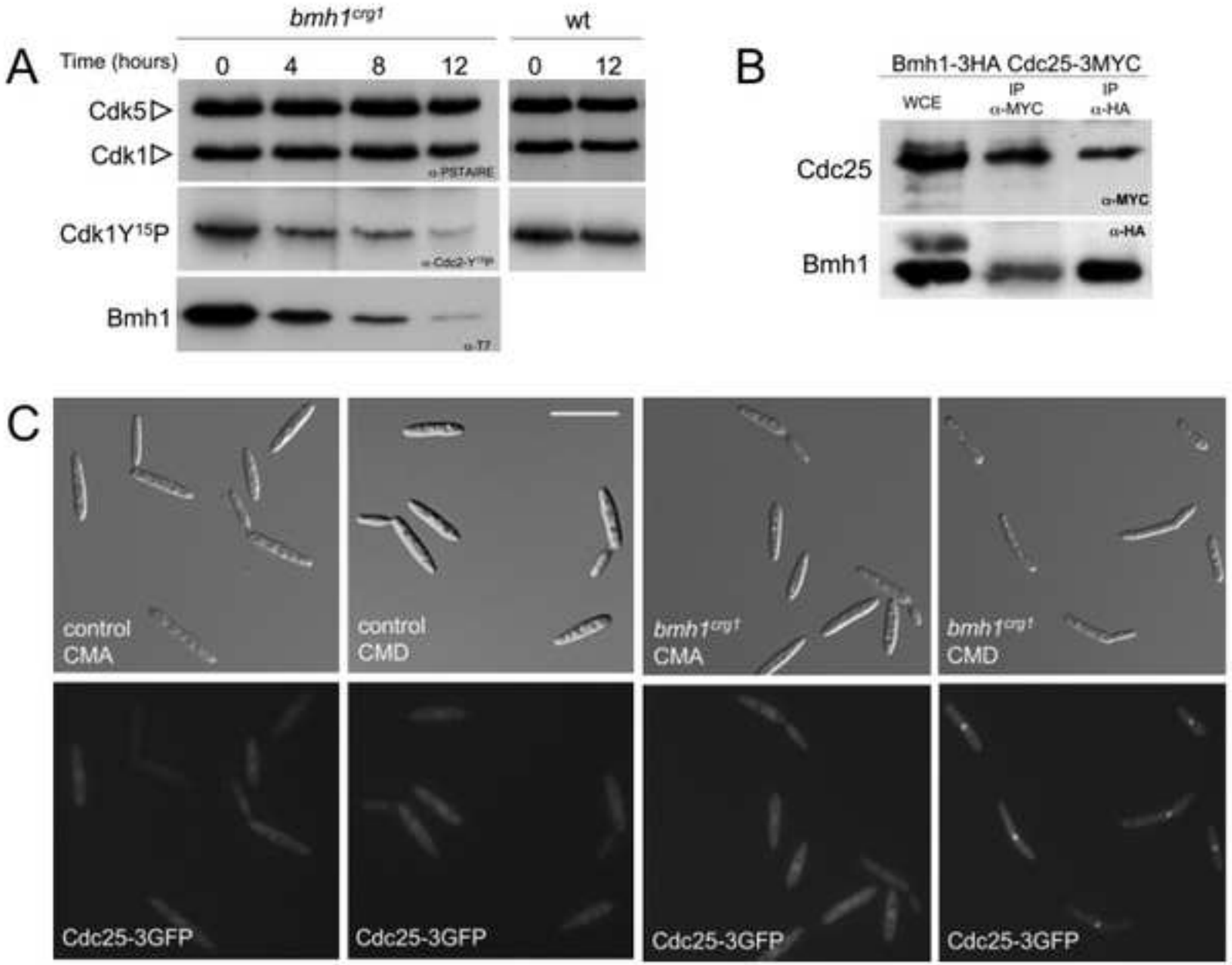

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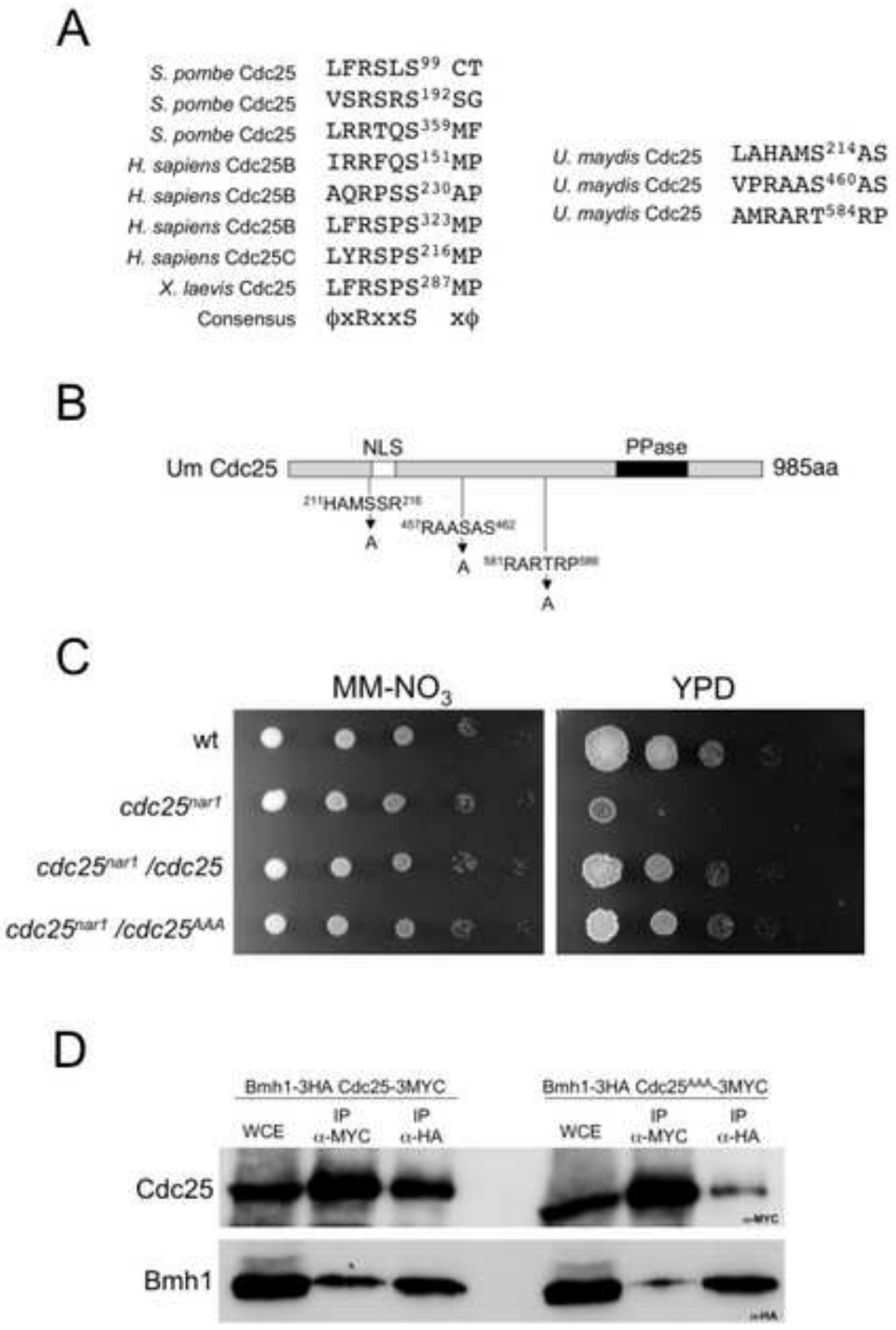

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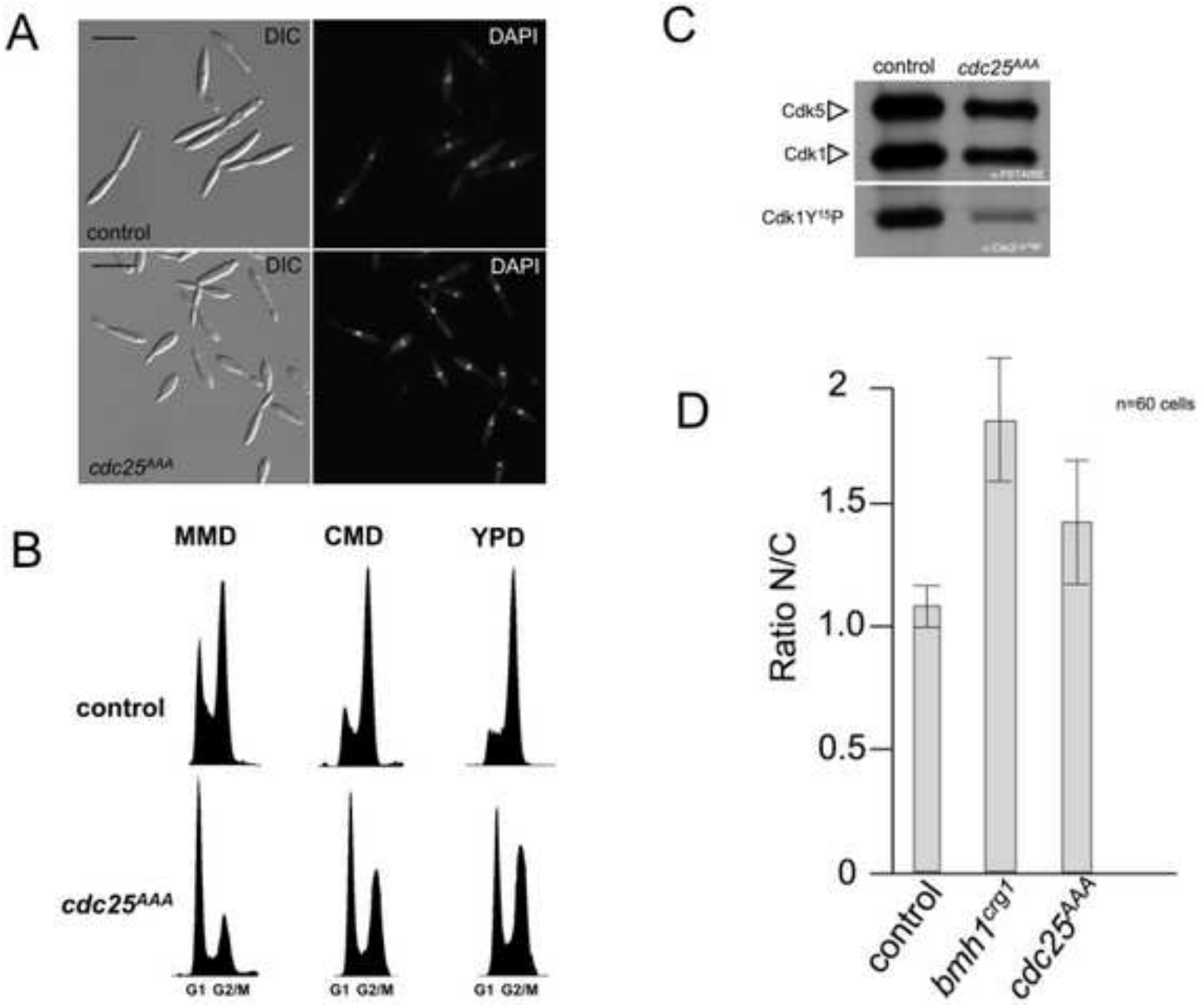

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