DNA-damage response in the basidiomycete fungus *Ustilago maydis* relies in a sole Chk1-like kinase

José Pérez-Martín

Departamento de Biotecnología Microbiana, Centro Nacional de Biotecnología-CSIC, Madrid, Spain

Address for correspondence:
Departamento de Biotecnología Microbiana, Centro Nacional de Biotecnología-CSIC, Campus de Cantoblanco-UAM, 28049 Madrid, Spain. Phone: +34-91-585-4704; FAX: +34-91-585-4506; e-mail: jperez@cnb.csic.es

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Abstract

Chk1 is a protein kinase that acts as a key signal transducer within the complex network responsible of the cellular response to different DNA damages. It is a conserved element along the eukaryotic kingdom, together a second checkpoint kinase, called Chk2/Rad53. In fact, all organisms studied so far carried at least one copy of each kind of checkpoint kinase. Since the relative contribution to the DNA damage response of each type of kinase varies from one organism to other, the current view about the roles of Chk1 and Chk2/Rad53 during DNA damage response is one of mutual complementation and intimate cooperation. However, in this work it is reported that *Ustilago maydis* -a phytopathogenic fungus exhibiting extreme resistance to UV and ionizing radiation- have a single kinase belonging to the Chk1 family but strikingly no kinases related to Chk2/Rad53 family are apparent. The *U. maydis* Chk1 kinase is able to respond to different classes of DNA damages and its activity is required for the cellular adaptation to such damages. As other described components of the Chk1 family of kinases, *U. maydis* Chk1 is phosphorylated and translocated to nucleus in response to DNA damage signals. Interestingly subtle differences in this response depending on the kind of DNA damage are apparent, suggesting that in *U. maydis* the sole Chk1 kinase recapitulates the roles that in other organisms are shared by Chk1 and the Chk2/Rad53 family of protein kinases.

1. Introduction

The fungus *Ustilago maydis* has been used as a model system for studying DNA repair for many years (1). A remarkable feature of *U. maydis* is its extreme resistance to UV and ionizing radiation (2). Strikingly, the molecular mechanisms behind such extreme resistance are poorly understood. It is thought that this resistance is due in large part to its homologous recombination system, as mutants defective in homologous recombination are extremely sensitive to UV and ionizing radiation (3-5). One characteristic of the homologous recombination system in *U. maydis* that distinguishes this organism from other model systems is its relative lack of complexity. For instance there is a single apparent Rad51 paralog (in comparison to several in *Saccharomyces cerevisiae* or mammals) and a noticeable absence of orthologs in numerous proteins important in recombination and DNA repair both in mammals and *S. cerevisiae* (1). In contrast, it is remarkable the presence of a BRCA2 ortholog, Brh2, absent in other fungal systems (5). However, apart of this, the analysis of the genome sequence focusing on proteins in homologous recombination produced little clues to the radiation resistance (1). Studies in other radioresistant systems such as the bacteria *Deinococcus radiodurans*, fueled the hypothesis that not a single mechanism provides a complete explanation for the radioresistance phenomenon. Instead, enhanced radioresistance seems to be the consequence of evolutionary process that has coordinated various mechanisms to produce a network of interacting molecular system series to protect the host cell against radiation damage (6).

One of the elements predicted to be important for radioresistance in organisms is the ability to monitor the presence of DNA damage during cell cycle progression. In eukaryotic cells, this sort of surveillance mechanism is an evolutionary conserved system called the DNA damage checkpoint pathway (7, 8). This pathway is composed of two cascades of signal transmission -the ataxia telangiectasia–mutated (ATM) and the ATM-related (ATR) signaling cascades- that simultaneously turn on DNA repair complexes and arrest cell division. Such a mechanism allows cells to repair damaged DNA before proceeding into mitosis (9, 10).

There are two main signals to be detected by DNA surveillance systems. The first one is the presence of single strand DNA tracts as a consequence of stalled replication forks in response to stresses such as the depletion of dNTP pools, inhibition of DNA polymerase, or as a consequence of the collision of replication forks with damaged DNA and /or aberrant DNA structures. The second major signal is the presence of double strand breaks in DNA. These signals are detected by sensors, which include two apical phosphoinositide 3-kinase-related kinases called ATM and ATR. ATM responds specifically to double-stranded breaks, whereas ATR primarily senses replication stress caused by a persistent block of replication fork progression (9, 11, 12). In both cases, ATM and ATR transmit signals by phoshporylating many substrates either directly or through the activity of two signal-transducing kinases, Chk1 and Chk2, which in turn phosphorylate key downstream effectors (13, 14).

The Chk1 and Chk2 kinases are primordial for the connections of DNA damage checkpoint pathway with the cell cycle machinery (15-17). Cell cycle

progression in eukaryotes is mediated by the activation of highly conserved family of protein kinases, the cyclin-dependent kinases (CDKs, 18). Activity of the mitotic CDK depends, among others, on the phosphorylation status of its catalytic subunit, Cdk1. Phosphorylation at conserved sites (Thr14 and Tyr15) prevent activation of Cdk1 avoiding transition to mitosis. Dephosphorylation and thereby activation of Cdk1 is carried out by the family of Cdc25 phosphatases (19). In most species (*S. cerevisiae* is an exception), DNA damage blocks G2/M transition because the inhibitory activity of Chk1 and Chk2 kinases on Cdc25 phosphatases (14, 20). Phosphorylation of Cdc25 phosphatase by Chk1 or Chk2 may results either in the sequestration of Cdc25 at the cytoplasm after binding to 14-3-3 proteins or in the targeting of Cdc25 for ubiquitin-mediated degradation. The outcome will depend on the organism as well as the kind of DNA damage (14, 21).

Chk1 and Chk2 kinases are structurally unrelated yet highly conserved in many organisms. However, several lines of evidence indicate functional differences among organisms. Chk1 seems to be more conserved as all organisms described so far have a single type of this kinase (17). In general, Chk1 is composed of a highly conserved N-terminal kinase domain, a flexible linker region, and a less conserved C-terminal region with undefined function (22). In some organisms, like mammalian and *Xenopus*, this C-terminal region has a negative role, and removal of this region produces a constitutively active protein (23). However, in other organisms such as *Schizosaccharomyces pombe*, the C-terminal end of Chk1 seems to have both positive and negative roles in the activity of the protein (24, 25).

The hallmark of Chk2 kinase is the presence in the same polypeptide of at least one fork-head-associated (FHA) domain and a serine-threonine kinase domain (26). Metazoan used to have a single Chk2 kinase, but in fungi there is not a clear rule. *S. cerevisiae* carries three kinases from this family: Rad53, Mek1 and Dun1 (27-29). In contrast, *Aspergillus nidulans* showed a single homologue, called ChkB (30). Just in the middle, in *S. pombe* and *Neurospora crassa* two Chk2-like kinases have been described, Cds1 and Mek1 (31, 32) and Mus-59 and Prd-4 (33, 34) respectively.

In an effort to understand further the molecular basis of the radioresistance in *U. maydis*, we decided to elucidate the connections between cell cycle and DNA damage response in this fungus. As a starting point, the identification of putative Chk1 and Chk2 orthologs in *U. maydis* was carried out. However, unexpectedly only a single ortholog of Chk1 kinase was obtained and no noticeable Chk2-like kinases were apparent. *U. maydis* Chk1 was required for radioresistance as Δ*chk1* mutant cells were largely sensitive to UV and ionizing radiation as well as other DNA insults. In spite of this major role, *U. maydis* Chk1 seems to behave as Chk1 paralogs in other organisms: it accumulates at the cell nucleus after DNA damage and phosphorylation at the C-terminal domain seems to be essential for *in vivo* activity. Therefore the data reported in this work mirror the apparent lack of complexity previously observed in functional elements involved in recombinational repair (1). An interesting open question is how the sole *U. maydis* Chk1 is able to manage the responses to different kind of DNA damages, a role executed in other systems by more than one kinase.

2. Materials and methods

2.1. Strains and growth conditions

Ustilago maydis strains are derived from FB1 background (35) and are listed in Table 1. Cells were grown in YPD or complete medium (CM) (36). Controlled expression of genes under the *crg1* promoter was performed as described previously (37). FACS analyses were described previously (38).

2.2. DNA and protein analysis

U. maydis DNA isolation was performed as previously described (39). Protein extracts, immunoprecipitations and Western analysis, were performed as described previously (37, 40). To detect the phosphorylated forms of Chk1, myc-tagged Chk1 proteins were immunoprecipitated using an anti-c-myc antibody (9E10, Roche) from cell extracts and subjected to SDS-PAGE in 8% acrylamide/0.1% bisacrylamide, pH 9.2 gels. Blots were incubated with anti-c-myc-horseradish peroxidase (Roche) and visualized using enhanced chemiluminescence (Renaissance ®, Perkin Elmer).

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). Plasmid pRU11-T7, to produce N-terminal T7 tags as well as expression under the control of P*crg1* promoter was constructed after insertion of the sequence encoding a T7 epitope as an *Nde*I-*Bam*HI fragment into pRU11 (41). Plasmid pBS-myc to produce C-terminal MYC protein

fusions was already described (42). 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 (39). Integration of the plasmids into the corresponding loci was verified in each case by diagnostic PCR and subsequent Southern blot analysis.

Deletion of *chk1* was done by gene replacement following the protocols of Kämper (43) and Brachmann et al. (44). Briefly, a pair of DNA fragments flanking the *chk1* ORF were amplified and ligated to a gene cassette encoding hygromycin resistance and flanked by *Sfi*I sites. The 5' fragment spans from nucleotide –1049 to nucleotide –1 (considering the adenine in the ATG as nucleotide +1) and it was produced by PCR amplification using the primers CHK1-2 and CHK1-3. The flanking 3' fragment was obtained after PCR amplification with primers CHK1-7 and CHK1-8 and spans from nucleotide +2008 to nucleotide +3148.

For C-terminal fusion of proteins to fluorescent markers, the adaptation of the *Sfi*I-dependent gene replacement strategy for C-terminal tag described in Becht et al. (45) was used. To produce Chk1-3GFP, 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 CHK1-14/CHK1-15 and spans from nucleotide +723 to nucleotide +1986. The flanking 3' fragment was obtained after PCR amplification with primers CHK1-7/CHK1-8 and spans from nucleotide +2008 to nucleotide +3148.

U. maydis Cut11 (um06416) is the corresponding ortholog to *S. pombe* Cut11, a well-known nuclear membrane marker (46). To produce Cut11-RFP, 5'- and 3'- fragments were digested with *Sfi*I and ligated to a cassette carrying the RFP-encoding gene as well as a nourseotricin resistance gene. The flanking 5' fragment was obtained after PCR amplification with primers CUT11-2/CUT11-3 and spans from nucleotide +746 to nucleotide +1908. The flanking 3' fragment was obtained after PCR amplification with primers CUT11-4/CUT11-5 and spans from nucleotide +1955 to nucleotide +2874.

To overexpress Chk1 and the derivate lacking the C-terminal half (Chk1Δ343), two DNA fragments were amplified from genomic DNA with the primers pairs CHK1-4/CHK1-29 and CHK1-4/CHK1-26. These fragments encode the full length ORF and a variant carrying the first 343 amino acids, respectively. The resulting fragments were digested with *Bam*HI and *Mfe*I and ligated to the corresponding sites in pRU11-T7. The kinase-dead alleles were produced by PCR-based mutagenesis (four-primer PCR with internal mutagenic primers). To obtain the full-length kinase-dead allele, the external primers were CHK1-4 and CHK1-29, while in the derivate lacking the C-terminal half the external primers were CHK1-4/CHK1-26. The internal primers carrying the mutation were CHK1-27/CHK1-28. The resulting fragments were ligated to the corresponding sites in pRU11-T7 as above.

To tag Chk1 with a 3myc epitope, a 1,98kb *Bam*HI-*Mfe*I fragment carrying the entire *chk1* ORF sequence without the stop codon was obtained by PCR amplification of *U. maydis* genomic DNA with the primers CHK1-4/CHK1-10 and this fragment was cloned into the corresponding sites in the plasmid pBS-MYC. A 0.7 Kb *Age*I-*Afl*II fragment from this plasmid was exchanged with the

corresponding 2.7 Kb *Age*I-*Afl*II fragment in the Chk1-3GFP cassette to produce a Chk1-MYC-Hyg cassette.

The alleles encoding alanine replacements of Chk1 were produced by PCR-based mutagenesis (four-primer PCR with internal mutagenic primers). In all cases the external primers were CHK1-4 and CHK1-10, and the construction of replacement MYC-tagged cassettes was similar to the construction of the wild-type myc–tagged allele (see above). Each alanine replacement carried associated the presence of a diagnostic restriction site. The internal mutagenic primer pairs as well as the associated restriction sites were: Thr336 to Ala336, CHK1-31/CHK1-32, *Xcm*I; Thr394 to Ala394, CHK1-18/CHK1-19, *Sty*I ; Ser448 to Ala448 CHK1-11/CHK1-12 *Msc*I; Ser472 Thr475 and Thr482 to Ala472 Ala475 and Ala482 CHK1-20/CHK1-21 *Fsp*I. The mutated alleles were 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. All the images in this study are single planes. Standard rodamine and GFP filter sets were used for epifluorescence analysis. The software used with the microscope was MetaMorph 7.1 (Universal Imaging, Downingtown, PA). Images were further processed with Adobe Photoshop 8.0.

2.5 Sequence analyses

Protein sequences of Chk1 and Rad53/Chk2 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 (47).

3. Results

3.1. U. maydis genome encodes a single Chk1 protein that is required for resistance to genotoxic insults

In a search for homologues of Chk1 and Chk2/Rad53 in the *U. maydis* genome database (http://mips.gsf.de/genre/proj/ustilago/) using BLAST analysis, only a single candidate to be a Chk1 homologue appeared (um03490). No putative Chk2/Rad53-like proteins were apparent. As proteins from the Chk2/Rad53 family are characterized by the presence of al least one single Fork-Head-Associated (FHA) domain and a Serine-Threonine Kinase (STK) domain, an additional search was performed looking for FHA-containing proteins in the genome database and then sorted the candidates for those carrying putative STK domains. From 8 putative proteins carrying at least a single FHA domain, no one carried an associated STK domain (Supplementary Table 1). As the *U. maydis* genome coverage was higher than 99.6% (48) it is considered highly unlikely that a homologue of Chk2/Rad53 family appears in *U. maydis*.

The putative *U. maydis* Chk1 protein consist of a 662 amino acids polypeptide (Fig. 1A). It shows a highly conserved N-terminal end carrying the kinase domain (for instance it shows e-values of $5e^{-55}$, $2e^{-53}$ and $7e^{-52}$ with the

corresponding kinase domain of Chk1 proteins from *Xenopus laevis*, *Homo sapiens* and *S. pombe* respectively), and a less-conserved C-terminal end where it is possible to detect several S/TQ motives. A dendrogram analysis of described members of Chk1 as well as Chk2/Rad53 families, located the putative *U. maydis* Chk1 in the Chk1 family. Moreover, Chk1 from *U. maydis* grouped in the branch formed by metazoan Chk1 kinases (Fig. 1B).

To support the idea that the putative Chk1 is involved in DNA damage response in *U. maydis*, a *chk1* null mutant was constructed. Cells carrying a deletion allele of *chk1* were viable, although they grew slightly slower than wild-type cells (not shown). These mutant cells were confronted to several genotoxic insults: UV irradiation, which makes DNA damages ranging from cyclobutane-pyrimidine dimers to DNA double strand breaks (DSBs); Hydroxyurea (HU), which inhibits ribonucleotide reductase and therefore affects replication by depletion of dNTPs, causing the accumulation of unreplicated forks; Methylmethane sulfonate (MMS), which induces DNA-alkylation; Phleomycine, a radiomimetic drug that causes DSBs on DNA; and IR irradiation, which generate DSBs. Δ*chk1* cells were more sensitive than wild-type cells to all the agents tested (Fig. 1C). Sensitivities to UV and IR irradiation were further quantitatively analyzed by making survival curves (Fig. 1D). The obtained results were compatible with the assays in plate, showing that Chk1 was required for radioresistance in *U. maydis*.

3.2 Cells defective in Chk1 function were unable to adjust cell cycle to the presence of DNA damage

The DNA damage checkpoint was originally defined as the pathway that promotes cell cycle delay in response to DNA damage. This delay is required to adapt the cell cycle to the presence of DNA damage, thereby providing more time for the repair of the damage (49). Cells defective on DNA damage checkpoint are unable to adjust their cell cycle and as a consequence they show genomic instability.

To analyze how *U. maydis* cell cycle adapts to the DNA damage and whether Chk1 is involved in this adaptation, wild-type and Δ*chk1* cells were confronted with sub-lethal concentrations of either HU (producing mainly the presence of unreplicated forks) or phleomycin (inducing DSBs). Asynchronous cultures of wild-type and mutant cells were analyzed for DNA content using Fluorescence/Activated Cell Sorter (FACS) analysis (Fig. 2A), as well as for cell morphology and nuclear distribution after DAPI staining (Fig. 2B).

Treatment of wild-type and Δ*chk1* cultures with 0.5 mM HU resulted in the accumulation of cells with 1C DNA content by 2 hours. However, while wild-type cells were able to proceed through S-phase, Δ*chk1* cells accumulated with a DNA content between 1C and 2C, and after an extensive incubation in the presence of HU (up to 8 h), cells carrying less than 1C DNA content started to accumulate (Fig. 2A, middle panels). At morphological level it was apparent in wild-type cultures the presence of cells carrying elongated buds and a single nucleus, a hallmark of G2/early mitosis stage in *U. maydis* (50), suggesting some delay in G2/M transition as it was expected after activation of the DNA damage checkpoint. In contrast in the mutant strain, aberrant cells carrying more than one bud were apparent, some of them sharing a single nucleus (see asterisk in Fig. 2B middle panels).

Treatment of wild-type cells with phleomycine also produces a similar morphological pattern as HU treatment, with the presence of cells carrying elongated buds and a single nucleus (i. e. G2/early mitosis cells, Fig. 2B right panel). At the level of DNA content it was apparent an accumulation of cells with 2C DNA content (Fig. 2A, right panel). In Δ*chk1* cells in contrast, no such accumulation of 2C DNA cells appeared and as incubation with phleomycine proceeds, cells carrying less than 1C DNA content accumulated. As it was observed in mutant cultures treated with HU treatment, cells with aberrant morphology were also apparent.

All together these data indicated that the absence of Chk1 impairs the ability of the cell cycle to be adapted to the DNA damage and therefore resulted in DNA instability, which can be traced by the presence of cells carrying less than 1C DNA content.

3.3 Chk1 arrest the cell cycle in G2 phase

Structural studies of human Chk1 kinase (22) as well as *in vitro* analysis with *Xenopus* Chk1 kinase (23) lead to a model in which the C-terminal domain of Chk1 blocks the N-terminal kinase domain and when phosphorylated by ATM/ATR, this inhibition is relieved. From this model it follows that expression of a truncated Chk1 lacking the C-terminal domain would produce a constitutively active protein. To test this idea, two versions of *U. maydis* Chk1, one encoding a full-length protein and the other encoding the first 343 residues carrying the kinase domain, were N-terminal tagged with T7 epitope and expressed under the carbon source-regulated promoter *crg1* (Fig. 3A). The presence of a N-terminal tag did not affect the ability of full-length protein

to complement a Δ*chk1* strain (not shown). The levels of expression of both proteins were similar under inducing conditions (Fig. 3B), however the effects on cells were dramatically different. While cells overexpressing the full-length Chk1 protein were indistinguishable from control, cells expressing the C-terminal truncated Chk1 were not able to produce colonies (Fig. 3C).

This growth defect was analyzed in liquid cultures. The different overexpression constructs were introduced in a strain carrying the Cut11-RFP fusion as a nuclear membrane marker (46). Since *U. maydis* undergoes an open mitosis (i. e. nuclear envelope disassembles in mitosis, 51), the use of this cytological marker helped to distinguish G2 from early mitosis. Cells overexpressing full-length Chk1 were indistinguishable from control cells (Fig. 3D). However, the totality of cells overexpressing the C-terminal truncated version of Chk1 was arrested as long budded cells having intact the nuclear membrane (Fig. 3D bottom panels). Cell DNA content in the different strains and growth conditions was also analyzed using FACS. The results obtained indicated that cells expressing the C-terminal truncated form of Chk1 accumulated with a 2C DNA content. Such accumulation was never observed neither in control cells nor in cells overexpressing the full-length protein (Fig. 3E). All together these data indicated that the expression of a C-terminal truncated form of Chk1 (presumably, a hyperactivated form) arrested cell cycle at G2.

In other systems it has been shown that the Chk1-induced G2 cell cycle arrest is a consequence of the phosphorylation of the mitotic inducer Cdc25 (52-54). Such a phosphorylation provides a binding site(s) for 14-3-3 proteins and causes Cdc25 to be excluded from nucleus (14, 55). To address whether the

G2 cell cycle arrest induced after overexpression of the N-terminal domain of Chk1 can be linked to this model, two experiments were carried out. First, a catalytically inactive allele (kinase-dead, KD) of Chk1 was produced by a lysine to alanine substitution at position 49 (equivalent to lysine 38 in *S. pombe* Chk1, 56). The full-length KD allele was unable to complement a Δ*chk1* strain and it acted as a dominant negative allele in a *chk1* wild-type background (not shown). When the N-terminal kinase domain of this KD allele was overexpressed, it did not produce cell growth arrest or cell elongation, even though it was expressed at similar level as the catalytically active form (Fig. 3B). This result indicated that kinase activity was required to induce cell cycle arrest.

For the second experiment, it was used a previously described allele of Cdc25 ($Cdc25^{AAA}$), which carries three serine to alanine mutations in the presumably phosphorylation sites for Chk1-like kinases. This mutant allele was impaired in checkpoint response to DNA damage and it was impaired in the ability to bind 14-3-3 proteins (57). When the N-terminal kinase domain of Chk1 was overexpressed in a strain carrying the $cdc25^{AAA}$ allele, no cell growth arrest was observed (Fig. 3B). This result was in agreement with the hypothesis that Chk1 targets Cdc25 in *U. maydis*.

3.4 Chk1 accumulates in the nucleus in response to DNA damage

Previous studies performed in *S. pombe* and mammalian Chk1 indicated that Chk1 accumulates in the nucleus in response to DNA damage (58, 59). To address whether this was the case for *U. maydis* Chk1, a strain carrying an endogenous Chk1-GFP fusion was constructed. This strain showed a wild-

type profile of sensitivity to agents inducing DNA damage, and therefore it was assumed that the C-terminal GFP tag did not affect the activity of the protein (not shown). As shown in fig. 4A, localization of Chk1 in untreated cells appeared to be pancellular, although occasionally cells carrying a clear nuclear fluorescence were observed (cells carried a Cut11-RFP fusion to determine the nucleus position). However, when cells were treated with DNA damage agents such as HU or phleomycin, an unambiguous nuclear accumulation of Chk1-GFP signal was observed in almost all cell population (Fig. 4B). Interestingly, some differences were noticed in the treatment with phleomycin versus HU. In cells treated with phleomycin (Fig. 4A, right panels) in addition to a nuclear localization, GFP fluorescence was also observed in punctuate cytoplasmic structures in variable number, usually one to two per cell, and frequently located nearby the nucleus. These cytoplasmic accumulations were rarely observed after HU treatment. As Chk1 has been shown in human cells to be involved in the spindle checkpoint and it can be located in kinetochores (60), one possibility was that the observed perinuclear localization of Chk1 in *U. maydis* corresponded to the spindle pole body. Unfortunately it was not the case, as these punctuate structures do not co-localized with γ-tubuline fused to RFP (Supplementary Fig. 1). The nature of these cytoplasmic accumulations remains unknown.

3.5 Chk1 is phosphorylated in response to DNA damage

Phosphorylation plays crucial roles in the regulation of cellular DNA-damage responses. In a number of systems, it has been shown that Chk1 protein is phosphorylated after DNA damage, and that this phosphorylation is linked to

its activation (61-63). Phosphorylation of Chk1 in response to DNA damage can be observed as a decrease in mobility on SDS-PAGE (56). In order to determine whether *U. maydis* Chk1 protein is phosphorylated in conditions of DNA damage, a C-terminal 3myc-tagged Chk1 allele was created and inserted at the native locus. The myc tag allows detection of Chk1 by immunoblot analysis and did not interfere with the ability of Chk1 to impart resistance to DNA damaging agents (not shown). Cultures from the strain carrying the Chk1-3myc allele were treated with HU or phleomycine for different times and then harvested for immunoprecipitation and immunoblot analysis. In the presence of either DNA damaging agent, Chk1 exhibited a retarded electrophoretic mobility (Figs. 5A, 5B). However, a slight difference was observed in the response to phleomycin with respect to HU. While in HU treated cells the change of Chk1 mobility is fast and remains unchanged for the treatment period (Fig. 5A), in phleomycine treated cells it was possible to detect after 2 hours a mobility shift that is further enhanced to a slower mobility form after longer incubations, suggesting successive modifications of the protein (Fig. 5B). These slow migrating forms were eliminated after λ-phosphatase treatment of immunoprecipitants indicating that the mobility shift was a consequence of phosphorylation (Fig. 5C).

3.6 Chk1 phosphorylation is required for in vivo activity

The Chk1 protein contains several SQ/TQ motifs that are consensus sites for phosphorylation by the ATM/ATR family of protein kinases (64). To determine whether phosphorylation of one or more of the conserved SQ/TQ motifs might be required for the above described mobility shift as well as for DNA-damage

response, mutations were introduced into *chk1* to change the relevant amino acid residue (serine or threonine) to alanine. The *chk1* mutant alleles were 3myc-tagged and integrated at the native locus. In a first approach single serine or threonine residues localized in the C-terminal domain were changed with the exception of the more C-terminal S/TQ cluster (Ser475, Thr 478 and Thr485) that was changed jointly ($chk1^{3A}$ allele). To evaluate the physiological importance of phosphorylation, the ability of wild-type and *chk1* mutants to survive exposure to UV light, phleomycine and HU was examined (Fig. 6A). Interestingly, T394A made cells more sensitive to HU, although not at the level of Δ*chk1*, and only slightly more sensitive to UV and phleomycine. It was also possible to detect some increase in HU sensitivity in S448A, although this mutant allele did not produce higher sensitivity to other DNA-damage inducing agents. Both mutations (T394A and S448A) were combined in a single allele and then they caused to cells degree of sensitivity to all DNA damages tested that was equivalent to that of Δ*chk1*.

The electrophoretic mobility shift of Chk1 in response to phleomycine and HU was analyzed for all above mutants. Mobility shift was largely eliminated in the double T394A S448A mutant in both conditions (Figs. 6B, 6C). However some of the single mutants behaved differently depending on the DNA damage agent. While in response to HU the T394A mutation abolished the mobility shift at levels comparable to double mutant, the S448A mutant protein was still able to shift although less efficiently than wild-type allele (Fig. 6B). Interestingly, in response to phleomycin both single mutant alleles behaved similarly, shifting the mobility to an intermediate position between the mobility displayed by wild-type allele in untreated and treated conditions (Fig. 6C).

Altogether, these results can be explained assuming that in response to phleomycin both residues (T394 and S448) has to be phosphorylated in order to produce an outcome, while in response to HU just T394 seemed to be enough, although phosphorylation of S448 helped the process.

3.7 Phosphorylation of Chk1 seems to be required for nuclear localization in response to DNA damage

Given that phosphorylation seemed to be required for Chk1 activation and that in response to DNA damage Chk1 accumulated in the nucleus, the relationships between phosphorylation and nuclear localization were investigated. To determine whether inability to be phosphorylated affected the nuclear accumulation of Chk1, the *chk1*$^{T394A\ S448A}$ allele was fused to GFP and its subcellular localization examined by microscopy in the presence of either HU or phleomycine (Fig. 7A). While control cells showed a clear nuclear accumulation of the fluorescent signal, the non-phosphorytable mutant failed to accumulate in the nucleus in response to DNA damage (Fig. 7B) suggesting that phosphorylation was important for the proper localization of Chk1 in response to DNA damage.

4. Discussion

Since the discovery of the DNA damage checkpoint, the original concept of two main pathways responding to specific signals showing strict dependency of Chk1 on ATR, and Chk2 on ATM, has been challenged. There are an increased number of reports in metazoan showing that these pathways are not each other isolated, and that there is continuous crosstalk among these

kinases. For instance, it has been reported phosphorylation/activation of Chk1 by ATM in response to ionizing radiation (65, 66) and phosphorylation of Chk2 in response to UV and HU independent of ATM (67). In addition, the fact that Chk1 and Chk2 perform partly redundant roles becomes evident from the spectra of their known substrates (68) most of which are shared by both kinases. Therefore, simple models of redundant or parallel functions performed by Chk1 and Chk2 in responses to genotoxic stress seems very unlikely. Instead, their emerging biological mission in metazoan is one of mutual complementation and intimate cooperation, a partnership where Chk1 operates as a workhorse while Chk2 contributes decisively only under circumstances that cause DSBs (21).

This already broad view has been challenged further by research performed in fungal systems. While for some fungal systems such as *A. nidulans* and *N. crassa* it seems apparent that Chk1- and Chk2-like kinases display an intense level of redundancy and cooperation (30, 34), in other systems is not. For instance, in *S. cerevisiae* Rad53 has a predominant role, and specific roles of Chk1 have been appreciated only recently (69). In contrast, in *S. pombe*, Chk1 is the main kinase and Cds1 (Chk2-like kinase) represents a backup system. The scenario is even more complex, as additional Chk2-like members were present in different fungal systems. While *A. nidulans* showed a single Chk2/Rad53-like kinase, *S. pombe* and *N. crassa* showed two different Chk2/Rad53 kinases and *S. cerevisiae* three members. In summary, it has become evident that even when the different components of the signal transmission pathway responsible of the DNA damage checkpoint are

conserved in all eukaryotic cells analyzed so far, the wiring of these components varies depending on the organism.

Paradoxically, in contrast to all this complexity, the results presented in this work indicated an apparent extreme simplicity in *U. maydis*. Only one homolog to Chk1 was found and no Chk2/Rad53-like kinases were noticeable from a search in the database. A similar search performed in other basidiomycete fungi indicated that they carried putative homologs to both family kinases (*Cryptococcus neoformans*: Chk1 XP572128.1 and Chk2/Rad53 XP567968.1; *Laccaria bicolor*: Chk1 XP001884536.1 and Chk2/Rad53 XP001877343.1; *Coprinopsis cinerea* (Chk1 XP001828231.1; Chk2/Rad53 XP001830159.1; unpublished observations).

The absence of Chk2/Rad53-like kinases in *U. maydis* implies that –unless additional uncharacterized kinases replace the role of Chk2/Rad53- Chk1 has to be competent to handle all the signals emanating from the different DNA damages. The analysis performed in this work indicated that Chk1 was required for the response to a variety of DNA genotoxic insults, and that its absence makes cells extremely sensitive to these insults. Furthermore, Chk1 was activated in response to the two main signals to be detected by DNA surveillance systems: the presence of double strand breaks in DNA (induced along this work after phleomycin treatment); and the presence of single strand DNA tracts as hallmark of replication stress (caused by HU treatment). Such Chk1 activation was monitored by two different ways: first as the accumulation of a GFP-tagged protein in the nucleus and second as the decrease in the mobility of a myc-tagged protein during electrophoresis as a surrogate marker for their phosphorylation-dependent activation. Interestingly some differences

were observed in the response to phleomycin versus HU treatment. At the level of subcellular localization it was notorious that after phleomycin treatment, in addition to nuclear accumulation of the fluorescent signal, several bright punctuate cytoplasmic structures were also observed. With respect to the decrease in electrophoretic mobility, whereas Chk1 was near-maximally shifted after short treatment with HU, phleomycin treatment produced a retarded band that is shifted to a sligthtly slower mobility band with further incubation time, suggesting that phleomycin-induced Chk1 phosphorylation occurred in at least two steps. These differences mirrored the data obtained with the use of mutant alleles carrying alanine substitution of the S/TQ phosphorylation sites. While the response to HU (measured as the ability to grow in the presence of the toxic as well as the electrophoretic mobility) can be impaired by a single mutation (T394A), to abolish the response to phleomycine it was required at least two amino acid changes (T394A, S448A). In summary these results suggested that most likely different upstream signals could result in different phosphorylation pattern/kinetics. No clue is provided in this work about how these differences are produced. It is worth to note that the genome of *U. maydis* carries two putative orthologs of the upstream kinases ATM and ATR (um15011 and um04151 respectively), suggesting that Chk1 in principle could to be able to receive signal from both kinases. Future studies will be dedicated to address this interesting possibility. To produce different pattern/kinetics of phosphorylation depending on the upstream signal could be crucial for the sole *U. maydis* Chk1 to be able to provide distinct responses depending on the stimulus. The mechanisms used by checkpoint kinases to transmit signals emanated from different classes of

DNA lesions are not clear. In other systems, Chk1 interacting proteins that promote damage specific responses have been identified. For instance, the BRCT motif-containing adaptor protein Crb2, which is required for *S. pombe* Chk1 activation is required for checkpoint signaling induced by UV, MMS and ionizing radiation but is dispensable when cells are exposed to HU (70, 71). The possibility that different signals resulted in different phosphorylation patterns and subsequently different responses has been addressed in *Xenopus*, where the checkpoint mediator protein Claspin is differentially phosphorylated depending on the kind of DNA damage (72). An appealing possibility in *U. maydis* Chk1 is that signal-specific phosphorylation pattern/kinetic dictates the downstream effectors, and thereby the kind of response. A phospho-counting mechanism involving SQ/TQ clusters that regulates different signaling outcomes have been recently demonstrated in the case of Rad53-Dun1 kinase cascade (73).

In conclusion, we believe that the simplicity of elements showed by the DNA-damage response in *U. maydis* could help to a better understanding of the mechanism required to determine how different DNA-damage signals are integrated by checkpoint kinases and converted into distinct responses. This work tries to be the founding basement for further approaches addressing these interesting questions.

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7. Legends to Figures

Fig. 1. *Ustilago maydis* Chk1 kinase

(A) Scheme of *U. maydis* Chk1. The N-terminal kinase domain is highlighted as well as the putative ATR/ATM phosphorylation sites (SQ/TQ).

(B) Dendrogram of characterized Chk1 and Chk2/Rad53-like proteins proteins. The tree was reconstructed using the ClustalW method (http://www.ebi.ac.uk/clustalw/). Numbers in the nodes indicate branch distances. The proteins utilized were *Homo* sapiens CHK1 (AAP36685.1) and CHK2 (EAW59756.1); *Xenopus laevis* CHK1 (AAF00098.1) and CDS1 (AAG59884.1); *Schizosaccharomyces pombe*; Chk1 (NP588070.1), Cds1 (NP587941.1) and Mek1 (NP594908.1); *Saccharomyces cerevisiae* Chk1 (EDN64882.1), Rad53 (EDV11072.1), Dun1 (NP010182.1) and Mek1 (NP014996.1); *Aspergillus nidulans* ChkA (XP663098.1) and ChkB (XP661883.1); *Neurospora crassa* MUS-58 (XP965249.2), MUS-59 (XP963844.1) and PRD-4 (XP964470.2); and *Ustilago maydis* Chk1 (XP759637.1).

(C) Sensitivity of cells lacking *chk1* gene in comparison to wild-type cells to different chemicals as well as IR and UV irradiation. Cell suspensions were spotted onto agar plates contained the indicated drugs (HU, hydroxyurea; Phleo, phleomycin; MMS, methyl methanesulfonate). For UV ad IR sensitivity, cells were irradiated at the indicated dose after being spotted. The spots were photographed after incubation for 2 days.

(D) Survival curves of wild-type and *chk1* defective cells against UV and IR irradiation. Cells were grown to late log phase, adjusted to a density of $2x10^7$

cells per ml and irradiated with UV or γ rays. Survival was determined by counting colonies visible after incubation for 2 to 3 days.

Fig. 2. Absence of Chk1 impairs the cell responses to DNA damage

(A) FACS analysis of wild-type and Δ*chk1* cells growing in liquid CMD in the presence of none (control), 0.5 mM hydroxyurea (HU) or 50 ng/ml phleomycin (Phleo). Time indicates hours in the presence of the compound.

(B) Morphology of cells incubated in the presence of hydroxyurea or phleomycin for 6 hours. Cells were stained with DAPI to locate nuclei. Note in the case of Δ*chk1* cells incubated in the presence of HU the presence of aberrant cells carrying more than one bud but a single nucleus (asterisk). All cells were shown at the same magnification (bar: 15 μm)

Fig. 3. Chk1 arrest cell cycle at G2 phase

(A) Scheme showing the constructions for the overexpression of full-length Chk1 as well as a C-terminal truncated version (Chk1Δ343). The proteins were N-terminal tagged with a T7 epitope and expressed under the control of *crg1* promoter, which is induced when cells are growing in the presence of arabinose as the sole carbon source and repressed in the presence of glucose.

(B) Levels of expression of the indicated proteins. The corresponding strains were grown in inducing conditions (complete medium plus arabinose, CMA) for 6 hours and cell extracts were obtained and subjected to immunoblot analysis using a commercial anti-T7 antibody. Similar amounts of total protein were loaded per sample (around 50 μg).

(C) Ability to produce colonies of the different strains growing in repressing condition (complete medium plus glucose, CMD) or inducing condition (CMA). Cell suspensions were spotted onto agar plates and incubated for 2 days at 28ºC.

(D) Morphology of cells expressing a C-terminal truncated Chk1 protein. Cells carrying an endogenous Cut11-RFP fusion to visualize the nuclear membrane and carrying either the construction overexpressing the full-length Chk1 or the C-terminal truncated version were grown for 6 hours in repressing (CMD) or inducing (CMA) conditions. All cells were shown at the same magnification (bar: 10 μm).

(E) FACS analysis of strains overexpressing the full-length Chk1 protein and the C-terminal-truncated version as well as control cells. Cells were incubated for the time indicated in CMD (repressing conditions) or CMA (inducing conditions).

Fig. 4. Chk1 is localized in the nucleus in response to DNA damage.

(A) Cells images of a strain carrying Chk1-3GFP and Cut11-RFP (a marker for nuclear membrane) fusions grown in CMD for 3 hours in the presence of none (control), 0.5 mM hydroxyurea (HU) or 50 ng/ml phleomycin (Phleo). All cells were shown at the same magnification (bar: 15 μm).

(B) Quantification of the cell response to DNA damage as the percentage of cells carrying a clear nuclear GFP fluorescence signal.

Fig. 5. *In vivo* phosphorylation of Chk1 in response to agents that induce DNA damage.

(A) Response to hydroxyurea. Cells carrying an endogenous Chk1-3myc allele were incubated in CMD for the indicated time in the presence of none (control) or 0.5 mM hydroxyurea (HU) Protein extracts were immunoprecipitated with a commercial anti-MYC antibody and immunoprecipitates were subjected to SDS-PAGE and immunoblotted with anti-MYC antibody.

(B) Response to phleomycin. Cells were treated as in (A) but using 50 ng/ml phleomycin (Phleo).

(C) Conversion of the modified form of Chk1 to the same mobility as the unmodified form after treatment with a protein phosphatase. Immunoprecipitates of cell extracts from cultures growing in the absence (control) or the presence of either hydroxyurea (HU) or phleomycin (Phleo) for 6 hours were incubated at 30ºC for 20 min in the absence (-) or presence (+) of lambda protein phosphatase (λ PPase) and were then subjected to immunoblot analysis.

Fig. 6. Chk1 phosphorylation is required for DNA damage response in *U. maydis*

(A) Cell suspensions of strains carrying the indicated *chk1* alleles were spotted onto agar plates contained the indicated drugs (HU, hydroxyurea; Phleo, phleomycin). For UV sensitivity, cells were irradiated at the indicated dose after being spotted. The spots were photographed after incubation for 2 days.

(B) Mobility shift of the different *chk1* mutants in response to hydroxyurea. Protein extracts from strains carrying the indicated *chk1* alleles (control is

wild-type myc-tagged allele) grown in the presence of 0.5 mM hydroxyurea for 6 hours, were immunoprecipitated with a commercial anti-MYC antibody and immunoprecipitates were subjected to SDS-PAGE and immunoblotted with anti-MYC antibody.

(C) as in (B) but growing the cells in the presence of 50 ng/ml phleomycin.

Fig. 7. Phosphorylation was required to localize Chk1 at the nucleus.

(A) (B) Cell images of a strain carrying either a wild-type Chk1-3GFP fusion protein (control) or a mutant allele unable to be phosphorylated in response to DNA damage agents ($chk1^{T394AS448A}$) after 3 hours of incubation in the presence of hydroxyurea or phleomycin.

(C) Quantification of the cell response to DNA damage as the percentage of cells carrying a clear nuclear GFP fluorescence signal.

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

Strain	Relevant genotype	Reference
FB1	*a1 b1*	35
UMP122	*a1 b1 Δchk1*	This work
UMP139	*a1 b1* P_{crg1}*:T7chk1*	This work
UMP167	*a1 b1* P_{crg1}*:T7chk1*^{K49A}	This work
UMP137	*a1 b1* P_{crg1}*:T7chk1Δ343*	This work
UMP168	*a1 b1* P_{crg1}*:T7chk1*^{K49A} *Δ343*	This work
UMP138	*a1 b1 cdc25*^{AAA} P_{crg1}*:T7chk1Δ343*	This work
UMP155	*a1 b1 cut11-RFP* P_{crg1}*:T7chk1*	This work
UMP156	*a1 b1 cut11-RFP* P_{crg1}*:T7chk1 Δ343*	This work
UMP158	*a1 b1 chk1-3GFP cut11-RFP*	This work
UMP124	*a1 b1 chk1-3myc*	This work
UMP163	*a1 b1 chk1*^{T336A}*-3myc*	This work
UMP175	*a1 b1 chk1*^{T394A}*-3myc*	This work
UMP176	*a1 b1 chk1*^{S448A}*-3myc*	This work
UMP166	*a1 b1 chk1*^{3A}*-3myc*	This work
UMP177	*a1 b1 chk1*$^{T394A\ S448A}$*-3myc*	This work
UMP178	*a1 b1 chk1*$^{T394A\ S448A}$*-3GFP cut11-RFP*	This work

Table 2. Oligonucleotides used in this study

name	Sequence
CHK1-2	5'TTAATTAATGCTGGTGATGACATCAGAGAGGGA3'
CHK1-3	5'GGTGGCCATCTAGGCCACTGCGTCTAGAGGTGTTGGTGG3'
CHK1-4	5'ACGGATCCATGACCATTCCAAAATCTTCAA3'
CHK1-7	5'ATAGGCCTGAGTGGCCCACACGCTTTTTCTCATCACCGC3'
CHK1-8	5'TTAATTAAATTGACCCTTGAGTGTACCGATGAA3'
CHK1-10	5'ATACAATTGCGTCGAGATGATTGTTTGCATGAT3'
CHK1-11	5'ATGTCACGTCGCTTGGCCATGGCCCAGCACATTTCATCGCGTC3'
CHK1-12	5'GACGCGATGAAATGTGCTGGGCCATGGCCAAGCGACGTGACAT3'
CHK1-14	5'AACGGCAAACCGTATCGTGGTGAGCCTGTCGAC3'
CHK1-15	5'GGTGGCCGCGTTGGCCGTCGAGATGATTGTTTGCATGAT3'
CHK1-18	5'TACCTGAGAACGTGTCCTTGGCTCAACCGGATGCAATTCT3'
CHK1-19	5'AGAATTGCATCCGGTTGAGCCAAGGACACGTTCTCAGGTA3'
CHK1-20	5'GGCGCAGCACAATTTGCGCAAGCGCTCAATCACTTTGCCCAAT3'
CHK1-21	5'ATTGGGCAAAGTGATTGAGCGCTTGCGCAAATTGTGCTGCGCC3'
CHK1-26	5'GCCAATTGTTAGTTCACTTCGACTATGATG3'
CHK1-27	5'CGGGGCATATGAGATGACCGCGATGGCTGCAACGCTATG3'
CHK1-28	5'CATAGCGTTGCAGCCATCGCGGTCATCTCATATGCCCCG3'
CHK1-29	5'CCGCAATTGCACACGACTCACGTCGAG3'
CHK1-31	5'CTGGTTCAAGCGTGCCAACGACCTCATGGCGCAGAAGGGGAAAT3'
CHK1-32	5'ATTTCCCCTTCTGCGCCATGAGGTCGTTGGCACGCTTGAACCAG3'
CUT11-2	5'TTAATTAACGCGTGGAATGATGCGAAGACTCTT3'
CUT11-3	5'TCTGGCCGCGTTGGCCCGCAAACACTCACCCAATTTGAGCTC3'
CUT11-4	5'ATAGGCCTGAGTGGCCATGTTGAGCATTCGCAATCAAATCCA3'
CUT11-5	5'TTAATTAACCGTACAGCACATTGGCCATCTATT3'

Figure 1

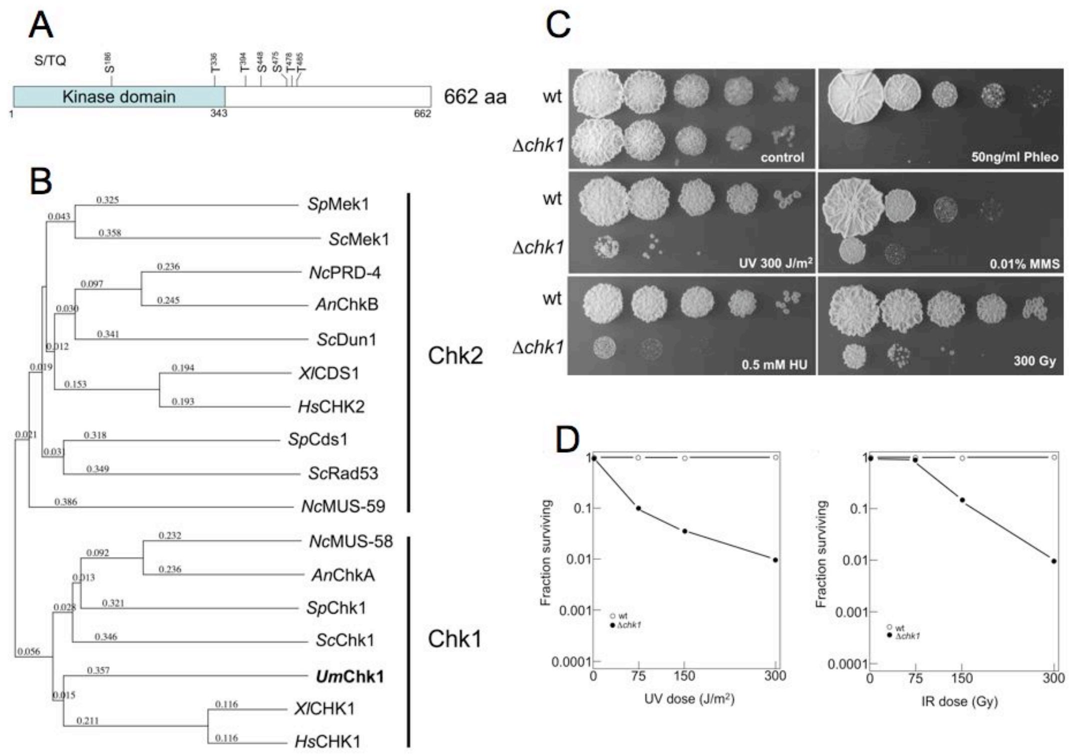


Figure 2.

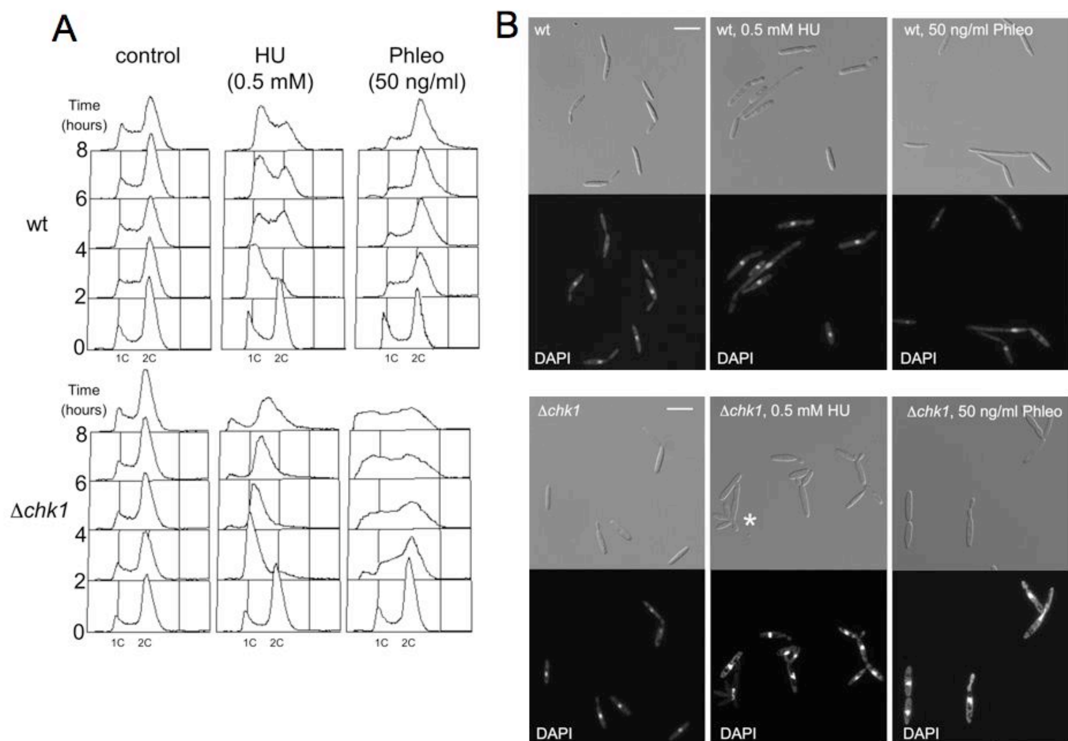


Figure 3

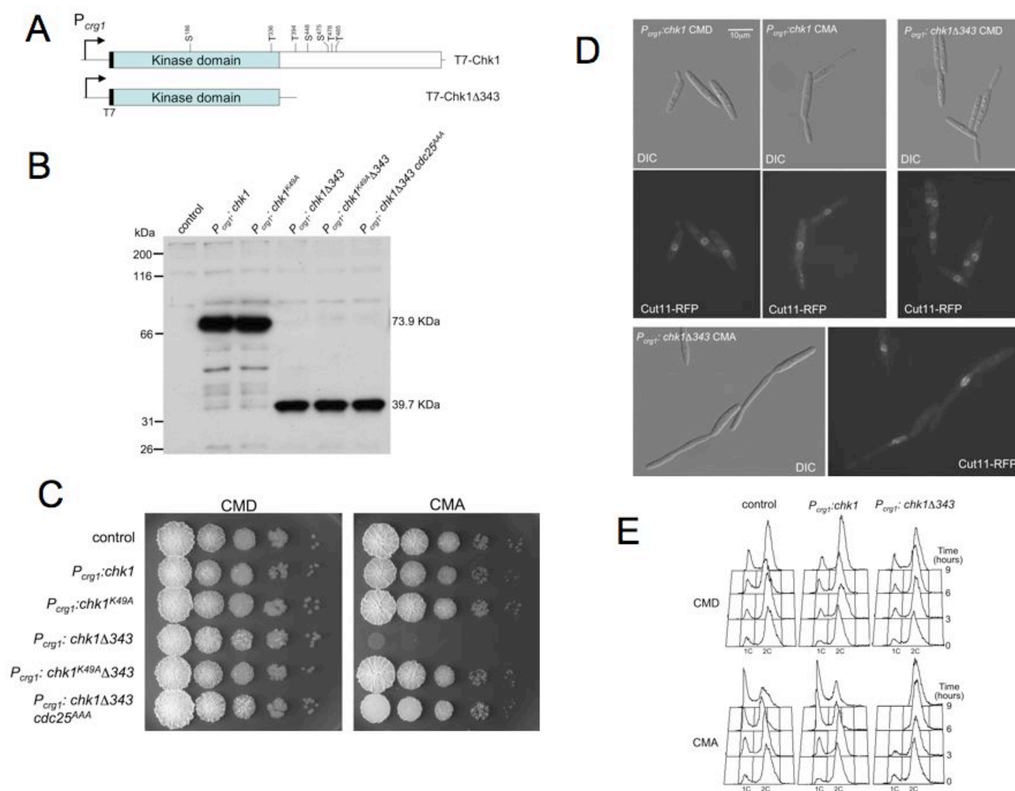
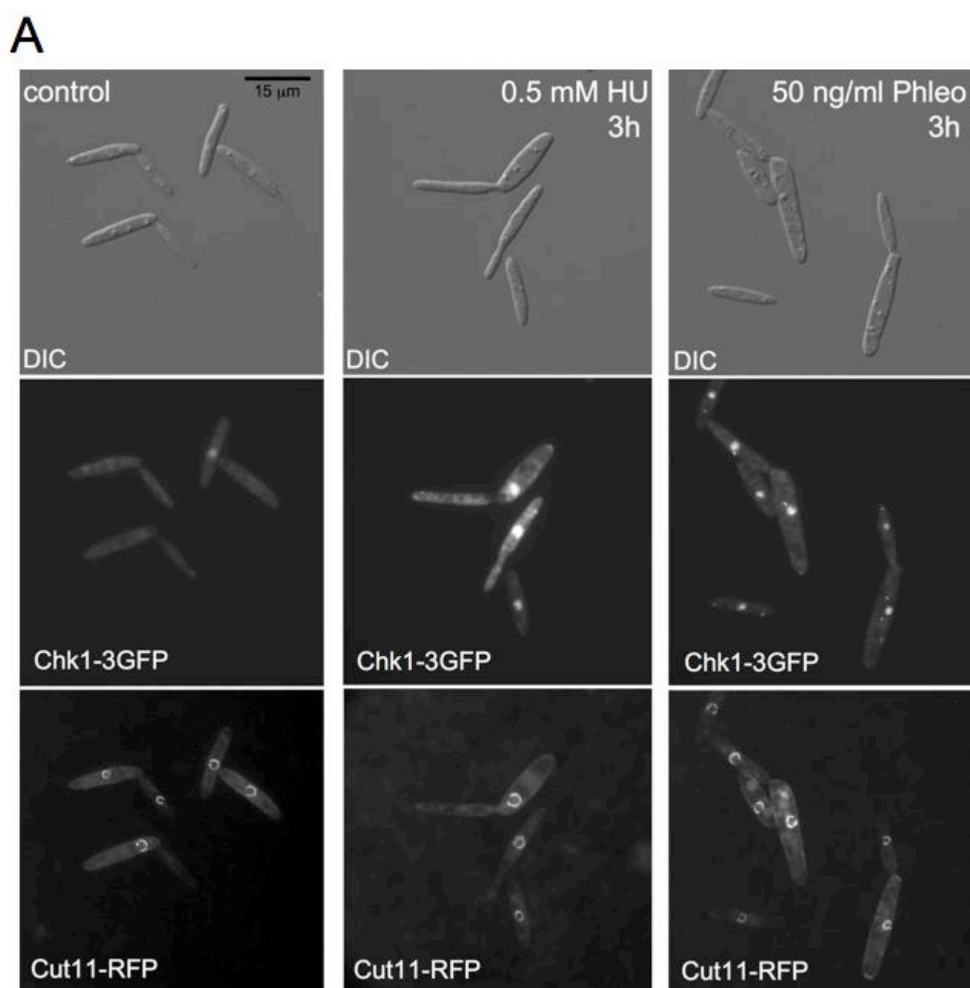


Figure 4



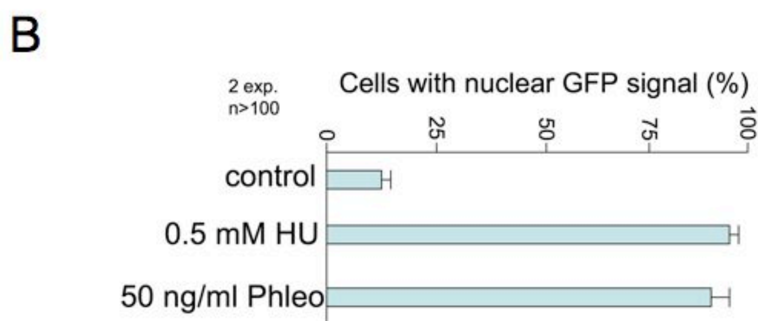


Figure 5

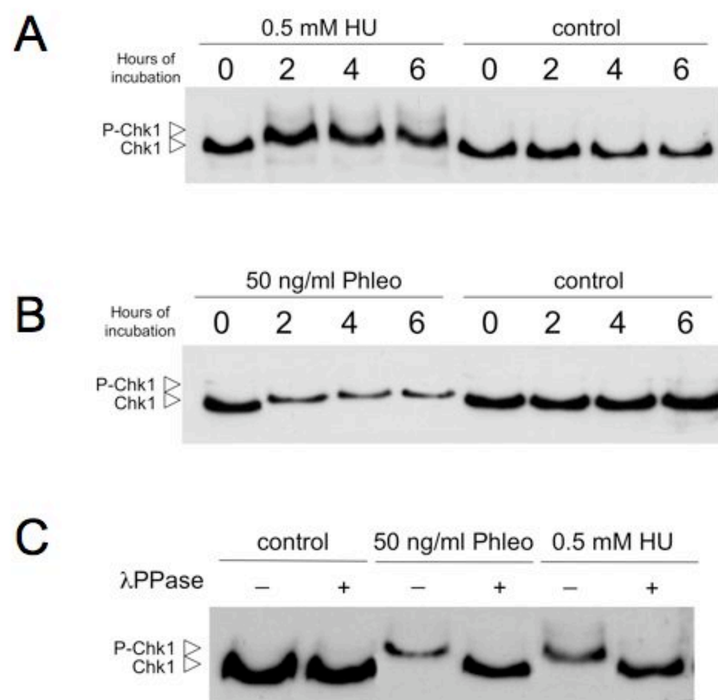


Figure 6

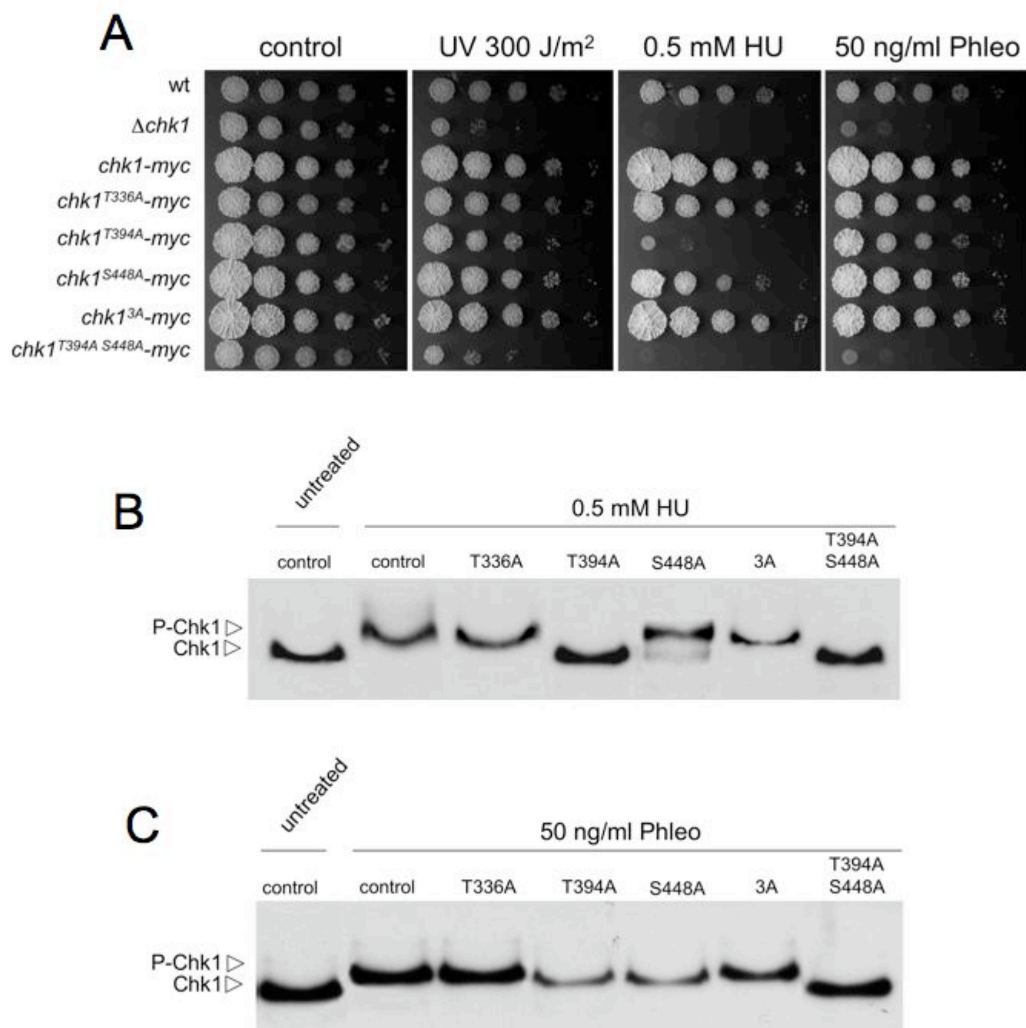
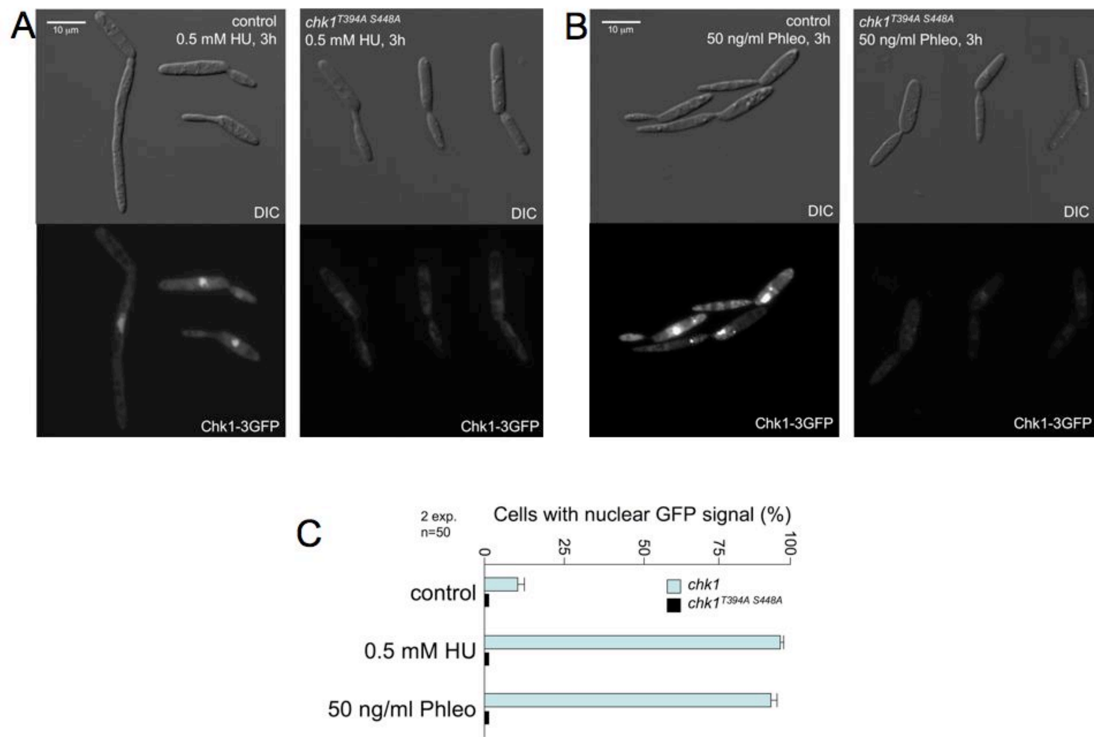


Figure 7

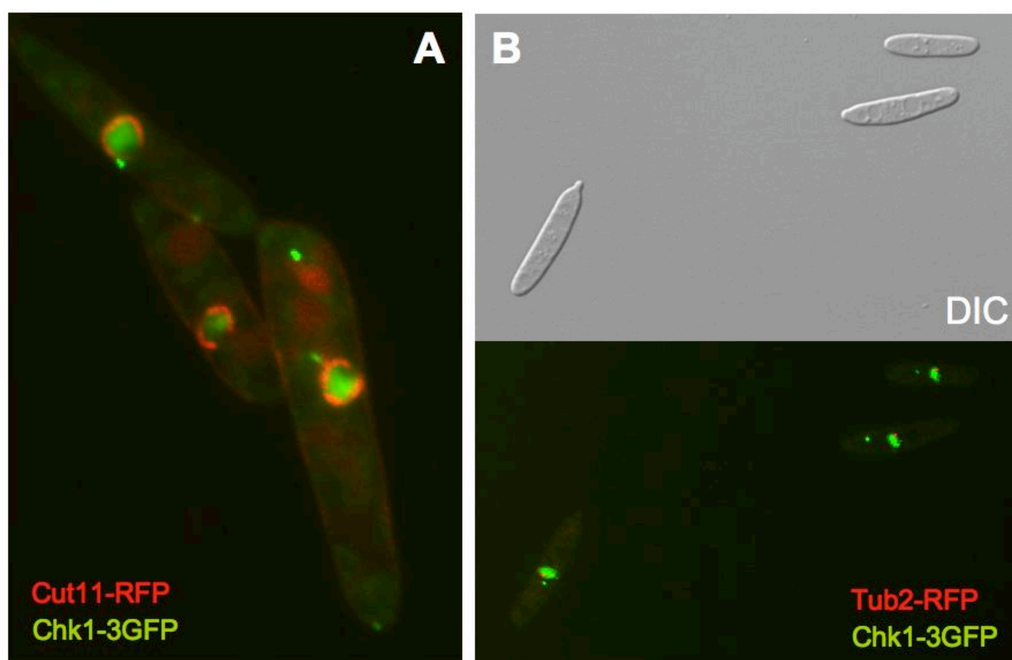


SUPPLEMENTARY TABLE 1

U. maydis proteins predicted to contain a Fork-Head-Associated (FHA) domain

UMDB number	Brief description
um00503	conserved hypothetical protein
um00857	probable KRE6 - glucan synthase subunit
um04391	conserved hypothetical protein
um05054	conserved hypothetical protein
um06251	Kinesin-3 motor protein
um10658	related to component of the spindle assembly checkpoint dma1
um11247	related to Smad nuclear interacting protein 1
um11592	conserved hypothetical protein

SUPPLEMENTARY FIGURE 1



(A) Strain UMP158, carrying a Chk1-3GFP and a Cut11-RFP fusion growing in CMD were treated during 3 hours with 50 ng/ml phleomycin.

(B) Strain UMP170 carrying a Chk1-3GFP and a Tub2-RFP fusion growing in CMD were treated during 3 hours with 50 ng/ml phleomycin.