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LAMMER kinase contributes to genome stability in *Ustilago maydis*

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Abstract

Here we report identification of the *lkh1* gene encoding a LAMMER kinase homolog (Lkh1) from a screen for DNA repair-deficient mutants in *Ustilago maydis*. The mutant allele isolated results from a mutation at glutamine codon 488 to a stop codon that would be predicted to lead to truncation of the carboxy-terminal kinase domain of the protein. This mutant (*lkh1*^{Q488②}) is highly sensitive to ultraviolet light, methyl methanesulfonate, and hydroxyurea. In contrast, a null mutant (*lkh1*Δ) deleted of the entire *lkh1* gene has a less severe phenotype. No epistasis was observed when an *lkh1*^{Q488②} *rad51*Δ double mutant was tested for genotoxin sensitivity. However, overexpressing the gene for Rad51, its regulator Brh2, or the Brh2 regulator Dss1 partially restored genotoxin resistance of the *lkh1*Δ and *lkh1*^{Q488②} mutants. Deletion of *lkh1* in a *chk1*Δ mutant enabled these double mutant cells to continue to cycle when challenged with hydroxyurea. *lkh1*Δ and *lkh1*^{Q488②} mutants were able to complete the meiotic process but exhibited reduced heteroallelic recombination and aberrant chromosome segregation. The observations suggest that Lkh1 serves in some aspect of cell cycle regulation after DNA damage or replication stress and that it also contributes to proper chromosome segregation in meiosis.

1. Introduction

Cells have elaborate mechanisms for dealing with damage to their genetic material. These include systems for recognizing DNA damage or aberrant DNA structures, and for responding to repair or neutralize the lesions and promote survival. To insure that integrity

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of the genome is maintained, the response to DNA damage must be precisely choreographed so that there is an integrated and coordinated procession of events. This involves coupling the genome surveillance, damage assessment, and repair systems with the cell cycle regulatory apparatus and chromosome architecture and chromatin remodeling machinery. An integral part of the entire network is the signaling circuitry that provides the means for communication among the various systems. A primary mode of signal transmission is achieved by cascades of phosphorylation events that modulate activity of DNA repair factors and govern cell cycle transitions [1, 2].

The phosphoinositide 3-kinase related Atm and Atr, are central factors in responding to DNA damage and aberrant structures formed during replication to halt the cell cycle and to coordinate repair processes [3-6]. Once these are activated they directly phosphorylate multiple targets including the effectors Chk1 and Chk2/Rad53, which in turn amplify and relay the signal to downstream targets [7-9]. Cyclin-dependent kinases (Cdks) are also involved in enabling repair by directing the repair pathway choice into the homologous recombination (HR[1]) system when cells enter S or G2 phase [10, 11]. This occurs by activating DNA resection at broken ends to generate ssDNA tails that are substrates for the Rad51 recombinase and at the same time destroying the flush-end DNA substrate required for repair by the nonhomologous end joining system [12]. Cdk activity also facilitates HR through modification of BRCA2 [13], which is a key regulator of Rad51 that is central to the cell's system for preserving genomic intergrity [14]. BRCA2 is also a target for checkpoint kinases Chk1 and Chk2, phosphorylation by which plays a critical role in dynamic association of Rad51 with BRCA2 [15].

Atr and Chk1 are also active in unperturbed normal cell cycle progression where the role is to regulate Cdk activity and to prevent late-origin firing [8, 16]. Initiation of replication is determined by Cdk activity, which is regulated negatively by phosphorylation of residue Tyr15 by the Wee1 kinase and positively by its dephosphorylation by Cdc25 phosphatase. Cdc25 is negatively regulated by Chk1, which in turn is stimulated by Atr.

LAMMER kinases constitute a subfamily of CDKs and have been reported to play roles in pre-mRNA processing [17, 18], cell cycle transitions [19], and global transcriptional regulation [20], but to date have not been reported to function in maintaining genomic integrity. Here we report identification of a LAMMER kinase homolog (Lkh1) of the basidiomycete fungus *Ustilago maydis* that came from a search for mutants defective in DNA repair and recombination. Our studies suggest Lkh1 functions in maintaining genome stability in *U. maydis* through an interplay with cell cycle regulators that is influenced by core homologous recombination components.

[1]The abbreviations used are: FACS, fluorescence activated cell sorting; HR, homologous recombination; HU, hydroxyurea; MMS, methyl methanesulfonate; UV, ultraviolet

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2. Materials and Methods

2.1 *U. maydis* strains and methods

Manipulations with *U. maydis*, culture methods, gene transfer procedures, survival after DNA damage, synthesis of diploids charcoal medium, and genetic crosses by mating *in planta* determinations have been described previously (see [21, 22] and references therein). Spot tests for survival were performed by diluting cultures to 2×10^7 cells per ml, then plating 10 μl aliquots of a series of sequential 10-fold dilutions on medium with or without genotoxins. Spot tests were repeated on independent isolates at least 3 times. UV irradiation was done using a Stratalinker 1800 (Bio-Rad). Doses indicated in the figures were the nominal settings taken from the instrument panel. However, using a dosimeter (Blak-Ray J225, UVP, Inc.) to monitor UV flux, we noted variability in the lamp output. Therefore the doses are considered approximations only. Allelic recombination at the *nar1* locus was measured by determining Nar^+ prototroph formation in diploids or in haploid progeny of meiotic products after germinating teliospores derived from matings [22]. Significance of differences observed was evaluated by Student's *t*-test. DNA content was measured by flow cytometry as described previously [22]. Microscopy was performed using a Nikon CF600 microscope. The *lkh1* gene was identified as entry um04543 in the annotated MIPS *U. maydis* database [see [23] and http://www.helmholtz-muenchen.de/en/ibis/institute/groups/fungal-microbial-genomics/] and was disrupted by standard methodology with cassettes expressing resistance to hygromycin (*hph*), geneticin (*nph*), or nourseothricin (*nst*) flanked by regions of homology proximal and distal to the open reading frame [24]. The *lkh1*^{Q488②} allele mimicking the allele isolated in the mutant screen was constructed by amplifying the *lkh1* gene from the Muv3 mutant then using this copy as a template for preparing a disruption vector. This vector was designed to include the mutant stop codon within the proximal homology flank region and to replace the kinase domain with a nourseothricin resistance cassette. *U. maydis* strains deleted of the following genes—*brh2* [21], *rad51* [25], *rec1* [26], *blm* [27], *atr* [28], *chk1* [29],-- were described previously, and derivatives were constructed as part of this study (Table 1). Self-replicating plasmids pCM973, pCM1019, pCM1030, and expressing the genes encoding Brh2, Dss1, and Rad51, respectively, under control of the glyceraldehyde 3-phosphate dehydrogenase (*gap*) promoter all relied on *hph* for selecting hygromycin resistance in *U. maydis*.

2.2 Mutant screen and gene determination

The mutant screen performed was described previously [21]. Briefly, colonies arising from suvivors after UV mutagenesis were screened for sensitivity to UV. Muv3 was found to be sensitive to UV, MMS, and HU. The defective gene was cloned by simultaneous complementation of the MMS and HU phenotype after introducing a genomic DNA library contained in a self-replicating vector into protoplasts, then plating on regeneration medium containing 0.01% MMS and 1 mM HU. Plasmid DNA recovered from resistant clones was evaluated by restriction enzyme digestion. The DNA sequences of the termini of complementing fragments were determined to delineate the boundaries of the cloned fragments, which were then matched to the *U. maydis* genome sequence in the MIPS database. Candidate genes identified by inspection of the genomic sequences were confirmed by subcloning and retesting for complementation. This was followed by

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amplification of the candidate open reading frame from genomic DNA of the Muv3 mutant using polymerase chain reaction, then determining the DNA sequence to establish identity of the inactivating mutation. The cDNA sequence of *lkh1* was determined after amplification of the gene from a cDNA library prepared from exponentially growing mitotic cells.

3. Results

3.1 Identification of Lkh1 from a screen for DNA repair mutants

We previously reported isolating DNA recombination and repair-defective mutants of *U. maydis* by screening colonies arising after radiation-induced mutagenesis for sensitivity to UV and methyl methane sulfonate (MMS) [21]. Among those identified were mutants defective in the genes encoding the BRCA2 ortholog Brh2 and the Rad51 recombinase. These exhibit extreme sensitivity to DNA damaging agents, are deficient in homologous recombination (HR), and are blocked in meiosis failing to yield meiotic progeny. In addition the cell morphology shows variation with a fraction of the population being markedly elongated presumably as a result of unrepaired DNA lesions triggering checkpoint activation and cycle arrest [30]. Another mutant obtained from that screen was Muv3 (*i.e.*, Muv phenotype, MMS and UV sensitive), which like the homologous recombination deficient mutants *brh2*Δ and *rad51*Δ, is highly sensitive to DNA clastogens and shows a similar variable cellular morphology with a fraction of the cells appearing distinctly elongated. But unlike the *brh2*Δ and *rad51*Δ mutants Muv3 is also highly sensitive to the DNA replication stressor hydroxyurea (HU) (Fig. 1).

We cloned the gene defective in Muv3 by complementation using a genomic DNA library in a self-replicating vector. After introducing the library into Muv3 cells and selecting for transformants, we screened those for ability to grow in medium containing MMS and hydroxyurea. Eight candidates were isolated. These could be divided into two classes on the basis of DNA damage sensitivity--one class with a wild type level of resistance to UV, MMS, and HU, while the other class with only partial activity (Fig. 2). From the five isolates of the first class one was arbitrarily chosen as representative clone 1, while from three isolates of the second class one was arbitrarily chosen as representative clone 2. The remaining candidates were not investigated further. Restriction enzyme analysis of the recovered plasmids from clones 1 and 2 revealed that the two cloned DNA fragments were unrelated. Sequence analysis of the 8 kbp genomic DNA fragment present in clone 1 revealed the presence of four genes. Predicted products included two uncharacterized proteins, a Hus5-related SUMO ligase and a LAMMER-family protein kinase homolog Lkh1. Given that Hus5 is known to play a role in repair of UV damage and resistance to HU in *Schizosaccharomyces pombe* [31], we initially surmised that mutation of this gene was responsible for the Muv3 phenotype. To check, we amplified and sequenced the Hus5 open reading frame from Muv3 genomic DNA, but found no mutation. Therefore, we subcloned the genes for Hus5 and Lkh1 from the cloned complementing DNA fragment and tested these individually for ability to complement Muv3. We found that introducing the subcloned gene for Lkh1 rather than Hus5 effectively restored resistance to UV, MMS, and HU, indicating that the *lkh1* gene was the likely candidate for the Muv3 mutant. This was confirmed by sequence determination of the *lkh1* gene from the Muv3 mutant in which was

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found a C1461T,C1462T dinucleotide change resulting in creation of a chain termination codon about half way into the open reading frame.

3.2 Lkh1 is a LAMMER family protein kinase

The structural gene for Lkh1, identified as open reading frame um4543 in the annotated Munich Information Center for Protein Sequences (MIPS) *U. maydis* database (Fig. 3A), is predicted to contain a small in-frame intron in the 5' region. However, computational analysis of the genomic sequence using a different program, the GenScan web server (http://genes.mit.edu/GENSCAN.html) does not predict an intron. This was confirmed from our determination of the cDNA sequence as isolated by PCR from a cDNA library of mitotically growing wild type cells, which revealed that the predicted intron is not spliced out. Therefore, the predicted protein expressed in mitotic cells is 728 amino acids in length with a highly conserved protein kinase domain located in the C-terminal residues 392-719 (Fig. 3B). An ATP binding motif is located at residues 398-421, the signature serine/threonine active site motif at 514-526, and the invariant motif EHLAMMERILG conserved in LAMMER kinases at 618-628 [32], this latter being essential for catalytic activity [33, 34]. The N-terminal region of the protein is quite extended and comprises almost half the length of the protein. It is highly divergent like other members of the LAMMER family with no recognizable sequence motif, but presumably directs the interplay with interacting partners. This part of the protein is referred to below as the regulatory region. The mutation responsible for the Muv3 phenotype results from sequence change at glutamine codon 488 to a termination codon. If this *lkh1* allele (*lkh1*^{Muv3}) were expressed the gene product (Lkh1^{Muv3}) would truncate between the ATP binding site and serine/threonine kinase active site yielding a polypeptide consisting of the extended regulatory region and ATP binding site, but deleted of the residues responsible for catalytic activity.

Deletion of the entire *lkh1* open reading frame resulted in a DNA damage sensitivity phenotype less severe than the *lkh1*^{Muv3} allele in regards to treatment with UV, MMS, and HU (Fig. 3C). This raised the possibility that an additional mutation at another locus in the original Muv3 isolate might contribute to the DNA damage sensitivity. To address this concern we engineered a mimic of the *lkh1*^{Muv3} allele from a wild type strain by introducing a termination signal (②) at the codon for Q488 and deleting the rest of the downstream sequence of the kinase domain. This mutant *lkh1*^{Q488②} has a phenotype like that of Muv3, *i.e.*, acute sensitivity to UV, MMS and HU. These findings show that the phenotype of the original Muv3 mutant arises from the point mutation in the *lkh1* gene and not from the additive effect of a second mutation in a different gene. It is possible that the Lkh1^{Q488②} polypeptide is expressed and that it interferes in some way with DNA repair or other processes. However, we found no experimental evidence to suggest that expressing the *lkh1*^{Q488②} allele either ectopically on a plasmid or in a diploid configuration with a wild type allele present conferred a dominant negative phenotype.

3.3 Core HR proteins suppress the DNA repair deficiency of Muv3

Sequence analysis of the 6 kbp fragment from the second class of complementing clones isolated from the screen revealed that the gene with partial activity in complementing the DNA repair phenotype of Muv3 encodes Brh2 (Fig. 2), suggesting that the action of Lkh1

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might be channeled through Brh2. This notion was investigated in the *lkh1*Δ and *lkh1*^{Q488②} alleles by introducing a self-replicating plasmid expressing Brh2 under control of a strong constitutive promoter. For simplicity we refer to this action below as overexpression, but caution that this is an assumption as we have not determined protein levels in the mutants. Suppression of the UV and HU sensitivity of the *lkh1*^{Q488②} allele by overexpressing Brh2 was particularly pronounced, while that of the *lkh1*Δ allele was marginal (Fig. 4). We did not investigate the other partially complementing candidate clones to determine if they too contained genomic fragments with the gene for Brh2. However, to check whether the suppression might be specific for Brh2, we tested if overexpression of other HR core components might also suppress the genotoxin sensitivity. Similar to the situation with Brh2, overexpressing Rad51, the recombinase governed by Brh2, or overexpressing Dss1, a regulator of Brh2, in the *lkh1*^{Q488②} mutant suppressed the sensitivity to UV, MMS, and HU on par with what was noted for Brh2. On the other hand, no suppression was noted upon overexpression of a number of other components of the HR system serving in functions upstream, downstream, or peripheral to the homologous pairing and strand invasion step (not shown). These included Mre11, Blm, ExoI, and Rad52. The lack of specificity for suppression in overexpressing a core HR component would appear to rule out the simple model that Lkh1 is an activator of Brh2.

3.4 Lkh1 operates in the response to DNA damage

We considered the possibility that Lkh1 might participate directly in the HR pathway. To investigate this idea we tested the highly UV sensitive *lkh1*^{Q488②} mutant allele for epistasis in UV sensitivity with a mutant defective in a core HR component, namely *rad51*Δ (Fig. 5A). In this case, sensitivity of the *rad51*Δ *lkh1*^{Q488②} double mutant to UV appeared additive, indicating independent modes of action in repair of damage. Further, the extreme sensitivity to HU of both the *lkh1* point mutant and deletion mutant was unlike the HU resistant phenotype exhibited by HR mutants such as *brh2*Δ or *blm*Δ suggesting a different pathway of action. On the other hand the HU phenotype was more reminiscent of mutants defective in DNA damage signaling and cell cycle checkpoint control such as *atr*Δ and *rec1*Δ (Fig. 5B), the latter deleted of a gene encoding a 9-1-1 checkpoint clamp component [35]. To investigate whether Lkh1 might function in some aspect of cell cycle progression, we tested the *lkh1*Δ and *lkh1*^{Q488②} alleles for epistasis in genotoxin sensitivity with *chk1*Δ (Fig. 5C). Chk1 is the single ATM/ATR effector kinase in *U. maydis* and is crucial for cell cycle checkpoint activation [28]. When the double mutant combinations of *lkh1*Δ and *lkh1*^{Q488②} with *chk1*Δ were tested for genotoxin sensitivity the results seemed somewhat contradictory. In the case of *chk1*Δ *lkh1*Δ it appeared that deletion of *lkh1* suppressed the sensitivity of *chk1*Δ to UV, MMS, and HU, suggesting that Lkh1 might function directly or indirectly to counterbalance the action of Chk1. On the other hand it was apparent with *chk1*Δ *lkh1*^{Q488②} that there was even greater sensitization than the *chk1*Δ single mutant. This finding is seemingly at odds with the epistasis in genotoxin sensitivity evident with the *chk1*Δ *lkh1*Δ complete deletions, but agrees with other observations on the *lkh1*^{Q488②} allele (see above) showing that it has a more severe phenotype than absence of the gene.

One of the roles of Chk1 in response to DNA damage is to adapt the cell cycle to such a challenge. To extend the above analysis, FACS was performed to assess cell cycle

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progression after treatment with HU. Exponentially growing cells were diluted into fresh medium with or without HU and samples were withdrawn every two hours for analysis of cellular DNA content (Fig. 5D). In wild type cells, treatment with HU induced a transient delay of cell cycle that was overcome with time. In the case of *chk1*Δ, cells accumulated in DNA content between 1C and 2C indicating that the cell cycle was arrested at S-phase. In the case of *lkh1*Δ and *lkh1*^{Q488②} the cells did not accumulate in S phase although the ability to progress in the cell cycle was not as efficient as wild type cells. This observation suggests that *chk1*Δ cells arrest in S after HU treatment as was previously observed [29], but that wild type, *lkh1*Δ, and *lkh1*^{Q488②} continue to cycle. It is interesting to note that like *lkh1*Δ cells a substantial portion of double mutant *chk1*Δ *lkh1*Δ cells with 1C DNA content also appeared to accumulate, supporting the finding that *lkh1*Δ alleviates the effects of HU poisoning in *chk1*Δ. DNA content of *chk1*Δ *lkh1*^{Q488②} cells after HU treatment was similar to *chk1*Δ cells indicating that the *lkh1*^{Q488②} allele is unable to suppress the HU phenotype of *chk1*Δ. These results suggest that Lkh1 provides some activity to counterbalance the action of Chk1 when DNA is damaged or when replication is stressed.

3.5 Lkh1 is required for proficiency in meiotic recombination and chromosomal segregation

Given the observed effect of expressing HR core components in rescuing the DNA damage sensitivity of the *lkh1* mutants, we asked whether Lkh1 contributes to homologous recombination proficiency. This was addressed by measuring gene conversion in mitotic diploid cells and in meiosis. For determining mitotic recombination compatible strains of the *lkh1*Δ or *lkh1*^{Q488②} mutant heteroallelic at the nitrate reductase locus (*nar1*) were mated to form diploids. These were tested for formation of Nar$^+$ recombinants by plating on selective medium containing nitrate as the sole source of nitrogen. Compared to the wild type control diploid the frequency of spontaneous or UV induced heteroallelic recombination was little different in the *lkh1*Δ or *lkh1*^{Q488②} mutant (data not shown). However, when meiotic recombination at *nar1* was measured in homozygous crosses of *lkh1*Δ or *lkh1*^{Q488②} the frequency of Nar$^+$ recombinants in the meiotic progeny was markedly reduced 30- to 40-fold ($p < 0.001$) compared to wild type (Fig. 6A).

It was also evident that chromosome segregation in the *lkh1*Δ and *lkh1*^{Q488②} crosses was abnormal. This was deduced from several lines of study. One was by plating sporidial cells from germinated teliospores on medium containing charcoal [21]. Cells heterozygous for the mating type *b* locus form white fuzzy colonies on charcoal medium (Fuz$^+$ phenotype) whereas true haploids form smooth gray colonies of uniform size [36]. Therefore, the appearance of Fuz$^+$ colonies is a measure of heterozygosity at the *b* locus and elevated frequency suggests a defect in chromosome segregation during meiotic cell division. In wild type crosses the frequency of Fuz$^+$ colonies is generally around 1% (Fig. 6B). By comparison, however, in the *lkh1*Δ or *lkh1*^{Q488②} cross the Fuz$^+$ frequency was respectively, 27% or 28% ($p < 0.001$). In addition there was obvious variation in colony size especially in the *lkh1*Δ cross suggestive of an abnormal chromosome complement. Furthermore, the parent strains were carrying different auxotrophic markers (*pan1-1* and *met1-2*). These ordinarily segregate randomly as they are unlinked, so the fraction of meiotic progeny from

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a wild type control cross that are prototrophs is about 25%. However, in the *lkh1*Δ cross the fraction of prototrophs was 53% indicating a deranged meiotic cycle and likely aneuploidy.

To examine chromosome status of the meiotic progeny in more detail we performed pulsed-field gel electrophoresis (CHEF) and FACS analysis of a number of randomly chosen Fuz^+ and Fuz^- colonies. We had established in an unrelated earlier study of meiotic products from *brh2*Δ × wild type heterozygous crosses that broken chromosomes could be readily detected by visual inspection of CHEF gels stained with ethidium bromide [21]. However, in 10 isolates tested from meiotic progeny of the *lkh1*Δ or *lkh1*^{Q488②} cross there was no sign of broken or aberrant chromosomes and the chromosomal DNA karyotype was not noticeably different from that of meiotic progeny from wild type crosses. We were unable to assess aneuploidy by this method because the resolution was too low. *U. maydis* has 23 chromosomes, several falling into groups of similar sizes. These cluster in similar mobilities during CHEF gel electrophoresis obscuring any difference and rendering resolution by inspection based on staining intensity too low to assess aneuploidy. However, there was evidence for aneuploidy by FACS. The FACS scans of the *lkh1*Δ and *lkh1*^{Q488②} samples analyzed showed a diploid or near diploid DNA content in every single isolate examined (Fig. 6C). This points to a possible requirement for Lkh1 in chromosome segregation in meiosis.

4. Discussion

Two main conclusions can be drawn from this investigation of Lkh1 in *U. maydis*. First, Lkh1 is required for maintaining genomic integrity in face of damage by DNA clastogens and DNA replication stress. Second, Lkh1 is necessary for proper chromosome segregation in meiosis.

A primary question posed at the outset of this investigation was what is the basis for the sensitivity of the *lkh1* mutants to DNA damage? The early finding that overexpression of Brh2 could suppress the *lkh1* mutant phenotype suggested to us that Lkh1 might participate directly in the HR pathway of DNA repair perhaps by phosphorylating Brh2 and activating it. This was an attractive notion, but one that we found no support for after several different lines of investigation. First, suppression of the *lkh1* mutant phenotype was not limited to overexpressing Brh2—Rad51 and Dss1 also were active in suppression. Second, the *lkh1* mutants are sensitive to the DNA replication stressor HU in addition to DNA clastogens, similar to mutants defective in the DNA damage response, but unlike bona fide HR mutants. Third, no epistatic interaction was observed in double mutant combinations of *lkh1* and *rad51*Δ suggesting Lkh1 functions outside of the recombinational repair process. Fourth, no deficiency was noted when heteroallelic recombination was measured in diploids. Collectively, these findings indicate that Lkh1 has no direct role in the homology-directed recombinational repair pathway in mitotic cells.

Given the similarities in phenotypes between the *lkh1*^{Q488②} allele and checkpoint mutants we considered the possibility that Lkh1 might contribute to cell cycle regulation. With focus on Chk1 as an effector in the DNA damage response and as an important regulator in cell cycle control and replication origin firing, we found an epistatic interaction in sensitivity to

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HU and DNA damaging agents in the *ckh1*Δ double mutant, namely that resistance of *ckh1*Δ could be restored by deletion of *lkh1*. This finding suggests that Lkh1 might function with Chk1 in a common circuit. Indeed, FACS analysis provides support for the notion that the common circuit is cell cycle control. *ckh1*Δ mutant cells challenged with HU were unable to pass through S phase, but deletion of *lkh1* restored the ability of these cells to continue cycling. Thus, it would appear that Lkh1 counterbalances the action of Chk1 in some way, possibly by modifying a secondary regulator of Cdk1. Recently it was reported that the Lkh1 ortholog in fission yeast activates Rum1, a CDK-inhibitor, to negatively regulate G1/S progression [19]. Possibly a similar mechanism is at play in regulation of the *U. maydis* cell cycle although it is not clear whether a Rum1 ortholog is present in *U. maydis.* We were unable to identify a Rum1-related protein in *U. maydis* by PSI-BLAST analyses run to convergence with multiple iterations.

The function of Lkh1 in meiosis would appear to extend beyond a cell cycle role. Meiotic allelic recombination is markedly reduced by the absence of Lkh1. In *U. maydis* meiosis is coupled to the parasitic stage of the fungal life cycle [37]. Dormant teliospores produced as the end products of the infection process contain diploid nuclei that have initiated meiosis but are arrested in meiotic prophase I most likely after recombination has been completed [22]. When plated on growth medium teliospores germinate with production of a promycelium and complete both meiotic divisions. The four haploid nuclei are distributed to separate compartments in the promycelium from which four sporidial cells representing the meiotic products bud off and enter mitotic growth. When homologous recombination fails due to the absence of the core DNA strand invasion function Rad51 or Brh2, teliospores form but are unable to germinate [21]. This is probably because the meiotic DNA-damage checkpoint cannot be deactivated due to the persistence of unrepaired DNA double strand breaks. From crosses with both the *lkh1* mutant alleles teliospores did form and were able to germinate, but allelic recombination was markedly reduced. This indicates that Lkh1 is probably not directly involved in core steps of eliminating DNA double-strand breaks, the lesions that activate the meiotic DNA damage checkpoint. Rather Lkh1 likely acts at some step prior to this, perhaps in chromosome pairing, DNA double-strand-break formation, or synaptonemal complex formation to influence the level of recombination. It is notable that *spo11*Δ mutants of *U. maydis* form teliospores that germinate to yield viable meiotic products. These are aneuploid and completely deficient in allelic recombination [22]. Nevertheless, a kind of aberrant meiosis can proceed even when DNA double-strand-break induction is absent and there is no recombination to direct accurate homolog disjunction. Perhaps Lkh1 serves to modify the activity of Spo11 or one of the constellation of factors that regulates its activity. Unfortunately there are no molecule tools available at this time to ascertain DNA double-strand-break processing in *U. maydis* meiosis. It should be noted that in Drosophila it has been reported that the Lkh1 homolog Doa serves in the regulatory program of the mitosis to meiosis switch [38]. It is possible this is related to the reduced meiotic recombination we observe.

The aberrant chromosome distribution apparent in *lkh1*Δ meiotic products as determined by high levels of heterozygosity at the *b* locus (Fuz^+ phenotype), non-Mendelian segregation of auxotrophic markers, and FACS analysis indicating near diploid DNA content of *lkh1*Δ

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meiotic products suggests a role for Lkh1 in meiotic chromosome segregation. The findings seem to fit best a model in which meiotic products result from a single division meiosis involving a mixture of reductional and equational segregation at meiosis I, but with without a second meiotic division. These characteristics closely resemble the phenotypic features observed in meiosis of the *U. maydis spo11*Δ mutant [22]. Thus, a disturbance in initiation of recombination by absence of Lkh1 might account for all the observed meiotic phenotypes. It will be interesting to determine whether depletion of Lkh1 results in a disturbance in meiosis in other eukaryotes.

A final issue in this study that warrants comment is the phenotype of the *lkh1*^{Q488②} allele in comparison with the *lkh1*Δ mutant. Why is it more sensitive to genotoxins, why is it suppressed to a higher degree by overexpressing HR core components, and why does it not rescue viability of *ckh1*Δ mutant cells challenged with HU in contrast to the *lkh1*Δ allele? We do not know the answer, but can only speculate that a truncated Lkh1 polypeptide is being expressed in the *lkh1*^{Q488②} mutant and that it must interfere with other processes, thus defining the difference between the alleles. It is interesting to consider the observation that expressing any one of the three HR components – Brh2, Dss1, or Rad51 – can suppress the *lkh1*^{Q488②} sensitivity to DNA damaging agents and HU. Since it has been demonstrated in mammalian cells that BRCA2 increases the pool size of Rad51 in the nucleus and that Dss1 blocks nuclear export signals present in both BRCA2 and Rad51 [39], the consequence of overexpressing any of these components could be to enhance the level of Rad51 in the nucleus, assuming this same paradigm holds true in *U. maydis*. In this context it is interesting to consider that Rad51 protects DNA from degradation when replication forks are stalled, in addition to its role in HR [40]. If the function of Lkh1 in mitotic cells is to contribute to cell cycle regulation, then perhaps the more severe phenotype of *lkh1*^{Q488②} mutant is due to interference by the truncated Lkh1 polypeptide with the process of restarting replication after fork stalling leaving forks more vulnerable to collapse.

In summary, our analysis of *lkh1* mutants in *U. maydis* demonstrates a novel role for this kinase in maintaining genomic integrity. Future studies will extend this initial insight and more fully assess the specific mechanisms by which Lkh1 contributes to this vital cellular function.

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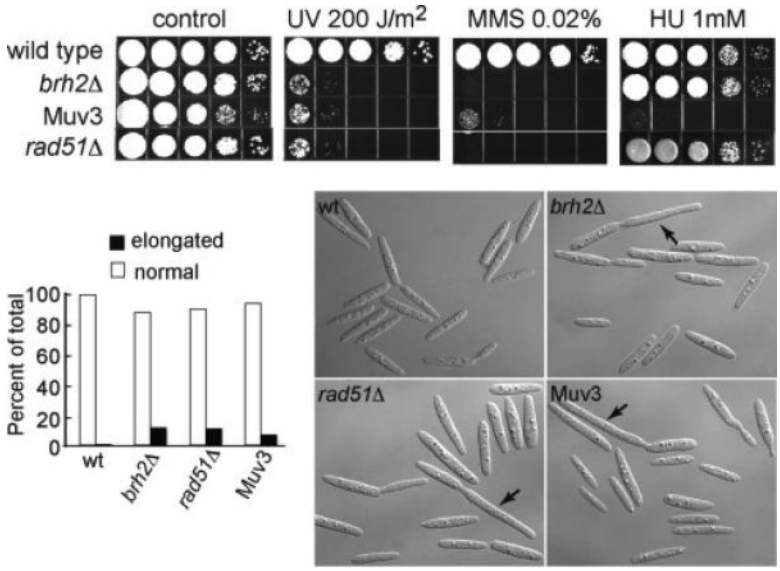

Fig. 1.

Phenotype of Muv3. The Muv3 mutant was tested for sensitivity to UV, MMS, and HU and compared with the *brh2*Δ and *rad51*Δ mutants. Spot tests were performed at least three times with independent isolates. Elongated cellular morphology evident in Muv3, *brh2*Δ, and *rad51*Δ mutants is illustrated in the micrographs by the arrows and was tallied as shown after counting 400-500 cells of each strain under a microscope using a hemocytometer.

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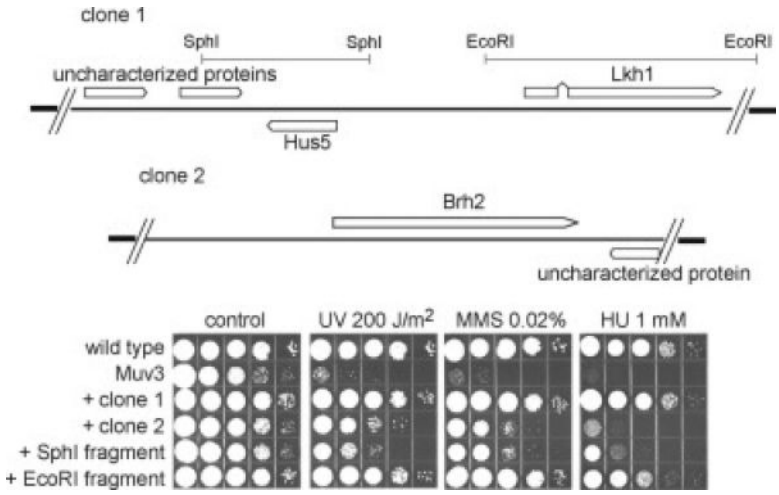

Fig. 2.

Cloning by complementation. Two classes of clones were obtained after introducing a genomic library into the Muv3 mutant. Representative clone 1 and clone 2 were compared for ability to rescue the sensitivity to UV, MMS, and HU. The two different DNA fragments isolated are illustrated with genes present according to the annotated MIPS database. Subclones were prepared from clone 1 with the restriction enzyme fragments indicated. Spot tests were performed on three independent isolates.

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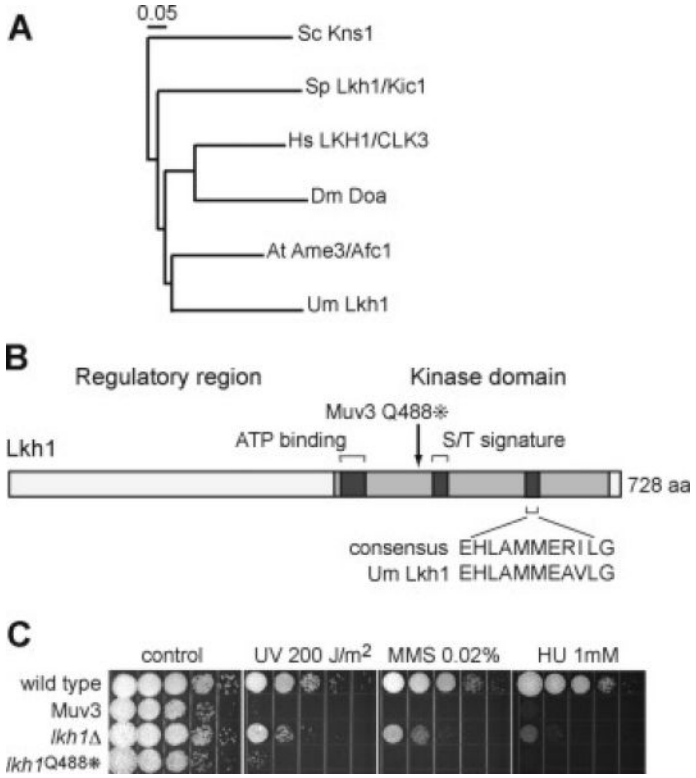

Fig. 3.

Lkh1 in *U. maydis*. A. Dendrogram of LAMMER kinases. The tree was constructed using the ClustalW method (http://embnet.vital-it.ch/software/ClustalW.html). Bar scale = 0.05 substitutions per amino acid. The proteins utilized were *Saccharomyces cerevisiae* (Sc) Kns1 (CAA97468); *Schizosaccharomyces pombe* (Sp) Lkh1 (CAD29835); *Homo sapiens* (Hs) LKH1/CLK3 (NP_001123500); *Drosophila melanogaster* (Dm) Doa (P49762); *Arabidopsis thaliana* (At) Ame3/Afc1 (P51568); *U. maydis* (Um) Lkh1 (KP966103). B. Lkh1 illustrated schematically with Kinase domain as gray bar and motifs for ATP binding, S/T kinase signature, and LAMMER signature motif in dark gray. The arrow indicates the site of mutation to a stop codon. C. Comparison of survival of *lkh1* alleles. Spot tests were performed at least three times.

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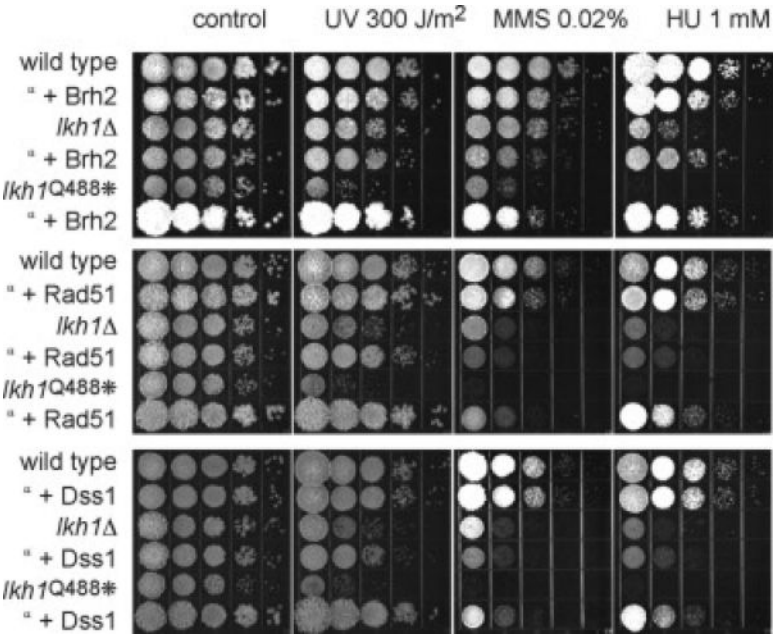

Fig. 4.

Suppression of genotoxin sensitivity by expression of core HR components. Plasmids expressing Brh2, Rad51, or Dss1 (e.g., indicated as + Brh2, etc.) were introduced into wild type, *lkh1*Δ, and $lkh1^{Q488k}$ strains. Survival was assessed after treatment as shown. Spot tests were performed at least three times with independent isolates.

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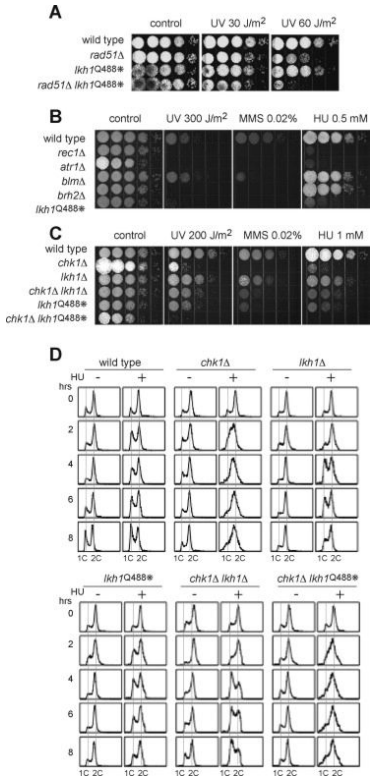

Fig. 5.

Epistasis and cell cycle effects. A. *lkh1*^{Q488k} was examined for epistasis with *rad51*Δ. B. Comparison of *lkh1*^{Q488k} phenotype with DNA damage checkpoint and HR mutants. C. *lkh1* alleles were examined for epistasis with *chk1*Δ. D. FACS scans were performed on exponentially growing cultures that were treated with 50 mM HU. Samples were removed for analysis at 2 hr intervals after addition of HU. Survival spot tests were performed at least three times with independent isolates. FACS analyses were performed with at least three different isolates for each strain and on two separate occasions.

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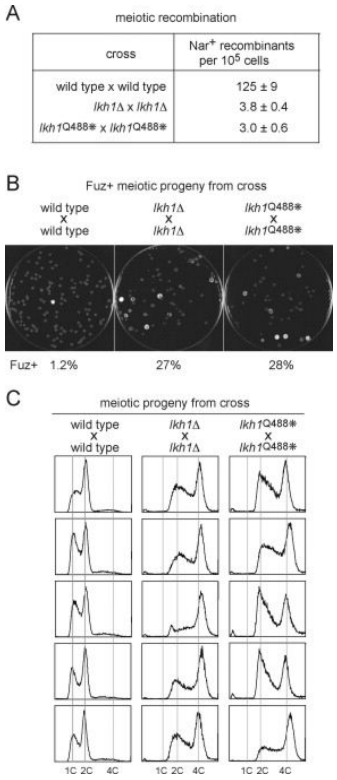

Fig. 6.

lkh1 meiotic phenotype. A. Heteroallelic recombination at *nar1* was measured in meiotic progeny from germinated teliospores. In the case of wild type Nar^+ recombinants were determined after10^5 cells were plated on each of two plates. In the case of the *lkh1* alleles Nar^+ was determined after 10^6 cells were plated on each of 4 plates. Standard deviations are shown. B. Meiotic progeny were spread on charcoal medium to measure Fuz phenotype. Representative plates are shown for wild type and *lkh1* alleles. 500-1000 colonies were tallied for each strain. Percentage of Fuz+ is shown underneath. C. FACS scan analysis of representative meiotic progeny from the crosses shown. For the *lkh1*Δ and $lkh1^{Q488k}$ alleles Fuz+ and Fuz^- colonies were chosen at random. FACS analysis was performed on two independent matings with 10 different meiotic product isolates for each genotype.

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Table 1

U. maydis strains

strain	genotype[a]	name
UCM350	*pan1-1 nar1-6 a1 b1*	wild type
UCM520	*met1-2 nar1-1 a2 b2*	wild type
UCM565	*brh2::nst pan1-1 nar1-6 a1 b1*	*brh2* Δ
UCM628	*rad51::nst pan1-1 nar1-6 a1 b1*	*rad51* Δ
UCM666	*rec1::nst pan1-1 nar1-6 a1 b1*	*rec1* Δ
UCM693	*blm::hph pan1-1 nar1-6 a1 b1*	*blm* Δ
MK59	*lkh1*$^{Q488@}$ *pan 1-1 nar1-6 a1 b1*	Muv3
MK59r51	*lkh1*$^{Q488@}$ *rad51::nst pan1-1 nar1-6 a1b1*	*rad51*Δ *lkh1*$^{Q488@}$
UCM810	*lkh1::nst pan1-1 nar1-6 a1 b1*	*lkh1* Δ
UCM813	*lkh1::nst met1-2 nar1-1 a2 b2*	*lkh1* Δ
UCM815	*lkh1*$^{Q488@}$*::nst met1-2 nar1-1 a2 b2*	*lkh1*$^{Q488@}$
UCM816	*lkh1*$^{Q488@}$*::nst pan1-1 nar1-6 a1 b1*	*lkh1*$^{Q488@}$
FB1	*a1 b1*	wild type
UCS1	*atr::hph a1 b1*	*atr* Δ
UMP122	*chk1::hph a1 b1*	*chk1* Δ
UCS84	*chk1::hph lkh1*$^{Q488@}$*::nst a1 b1*	*chk1*Δ *lkh1*$^{Q488@}$
UCS87	*chk1::hph lkh1::nst a1 b1*	*chk1*Δ *lkh1*Δ

[a] *pan*, *met*, *nar*, *a* and *b* indicate requirement for pantothenate, methione, inability to reduce nitrate, and mating type loci, respectively. *::* indicates deletion and replacement by a drug resistance marker.