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The *Aspergillus nidulans sldI*^{RAD50} gene interacts with *bimE*^{APC1}, a homologue of an anaphase-promoting complex subunit

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Summary

The Mre11–Rad50–Nbs1 protein complex has emerged as a central component in the human cellular DNA damage response, and recent observations suggest that these proteins are at least partially responsible for the linking of DNA damage detection to DNA repair and cell cycle checkpoint functions. We have identified *Aspergillus nidulans sldI1444D* mutant in a screen for dynein synthetic lethals. The *sldI*^{RAD50} gene was cloned by complementation of the sporulation deficiency phenotype of this mutant. A transversion G→C at the position 2509 (Ala-692-Pro amino acid change) in the *sldI1444D* mutant causes sensitivity to several DNA-damaging agents. The mutation *sldI1* occurs at the *CXXC* hinge domain of Rad50. We have deleted part of the coiled-coil and few amino acids of the Rad50–Mre11 interaction region and assessed several phenotypic traits in this deletion strain. Besides sensitivity to a number of DNA-damaging agents, this deletion strain is also impaired in the DNA replication checkpoint response, and in ascospore viability. There is no delay of the S-phase when germlings of both *sldI*^{RAD50} and *mreA*^{MRE11} inactivation strains were exposed to the DNA damage caused by bleomycin. Transformation experiments and Southern blot analysis indicate homologous recombination is dependent on *scaA*^{NBS1} function in the Mre11 complex. There are epistatic and synergistic interactions between *sldI*^{RAD50} and *bimE*^{APC1} at S-phase checkpoints and response to hydroxyurea and UV light. Our results suggest a possible novel feature of the Mre11 complex in *A. nidulans*, i.e. a relationship with *bimE*^{APC1}.

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Introduction

Accumulation of mutations will eventually lead to genomic instability and, consequently, carcinogenesis in higher organisms. Cell cycle checkpoints and DNA repair are the primary defences against genomic instability. If the DNA damage is extensive, apoptosis provides a mechanism to eliminate cells with high mutation potential (Qin and Li, 2003). DNA double-strand breaks (DSBs) are the most dangerous form of DNA damage. Chromosomes are left broken and the cells are driven towards death if a DSB is not repaired. In addition, chromosome translocations and genome rearrangements result from improper repair of DSBs. There are at least two independent pathways that have evolved to repair DSBs: homologous recombination (HR) and non-homologous DNA end joining (NHEJ) (D'Amours and Jackson, 2002). The Mre11–Rad50–Nbs1 complex has emerged as a central component in the human cellular DNA damage response and has been implicated in the repair of DSBs by both pathways in yeast and mammals (Petrini *et al.*, 1997). Recent observations suggest that these proteins are at least partially responsible for the linking of DNA damage detection to DNA repair and cell cycle checkpoint functions (Petrini, 1999). In humans, loss of NBS1 gene is associated with the Nijmegen breakage syndrome (NBS), a rare autosomal recessive disorder that belongs to the group of inherited human chromosomal instability syndromes that includes Bloom's syndrome, Fanconi's anaemia and ataxia telangiectasia (AT). All of these disorders are characterized by spontaneous chromosomal instability, immunodeficiency and predisposition to cancer, but have distinct cytogenetic features and sensitivities to specific DNA-damaging agents (for reviews, see Digweed, 1993; Shiloh, 1997; Stewart and Elledge, 2002). The NBS1 gene product, nibrin/p95, is part of the Mre11/Rad50 nuclear foci that form at sites of DSBs (Carney *et al.*, 1998). The Rad50–

Mre11–Nbs1 complex possesses manganese-dependent single-stranded DNA endonuclease and 3′ to 5′ exonuclease activities (Trujillo *et al*., 1998). In humans and in yeast, Mre11, Rad50 and Nbs1/Xrs2 assemble into large complexes. These proteins also appear to play a role in telomere maintenance. In addition, they are implicated in the cell's checkpoint response to the presence of DSBs (D'Amours and Jackson, 2002). During meiosis in *Saccharomyces cerevisiae*, these three proteins are required not only for resection of DSBs, but also to create the meiotic DSBs (Petrini, 1999). Studies in mammalian and yeast cells have established that the Mre11 complex controls the ATM/Tel1 signalling pathway (Kastan and Lim, 2000; Petrini, 2000; Usui *et al*., 2001; D'Amours and Jackson, 2002). ATM activation correlates with autophosphorylation on Ser^{1981} (Bakkenist and Kastan, 2003), and this phosphorylation requires functions of the Mre11 complex (Carson *et al*., 2003; Uziel *et al*., 2003). It is possible that the Mre11 complex modulates the substrate recognition of ATM by direct interaction (Lee and Paull, 2004). In addition, the Mre11 complex controls the accumulation of ATM at DSB lesions (Kitagawa *et al*., 2004). Recently, Nakada *et al*. (2004) have shown that the Mre11 complex functions together with exonuclease I (Exo I) in activation of $Mec1^{ATR}$ signalling pathway after DNA damage and replication block. During DSB- and UV-induced checkpoints, the Mre11 complex and Exo I collaborate in producing long single-stranded DNA tails at DSB ends and promote Mec1 association with the DSBs.

During DSB repair, Rad50, the large coiled-coil ATP-binding cassette (ABC) ATPase, a structural maintenance of chromosome (SMC) protein related, uses ATP to bind and bridge DSB, and to facilitate DNA end processing by the nuclease Mre11 (Hopfner *et al*., 2002a; Hopfner and Tainer, 2003). N- and C- terminal ATPase segments of Rad50 assemble into a functional ABC-type ATPase domain at the end of an antiparallel *coiled-coil* region (Connelly *et al*., 1998; Anderson *et al*., 2001; Hopfner *et al*., 2001). Hopfner *et al*. (2002b) presented a crystal structure of the Rad50 *coiled-coil* region that reveals a dimer interface at the apex of the *coiled-coil* in which pairs of conserved *Cys-X-X-Cys* (*CXXC*) motifs form interlocking hooks that bind one Zn^{+2} ion. The probable DNA binding site of Rad50 is in the ABC-ATPase domain that has ATP-stimulated DNA-binding activity. Mre11 binds to the *coiled-coil* domain near the ATPase domain of Rad50 (Anderson *et al*., 2001; Hopfner *et al*., 2001). It has been suggested that the Mre11 exonuclease function is directly controlled by ATP-dependent conformational changes of the Rad50 ATPase domain (Hopfner *et al*., 2002a). The Rad50 hook is functional, because mutations in this motif confer radiation sensitivity in yeast and disrupt binding at the distant Mre11 nuclease interface (Hopfner *et al*., 2002b).

We have been using *Aspergillus nidulans* as a model system to genetically characterize the cellular response to DNA damage (Bruschi *et al*., 2001; Fagundes *et al*., 2003; 2004; Semighini *et al*., 2003; for a review, see Kafer and May, 1998; Goldman *et al*., 2002; Goldman and Kafer, 2004). Previously, we isolated camptothecin-sensitive mutants of *A. nidulans* and the $scaA^{NBS1}$ gene, which is the apparent homologue of the human nibrin gene, was cloned by complementation of the camptothecin-sensitive *scaA1* mutation (Bruschi *et al*., 2001). Semighini *et al*. (2003) have used the $scaA^{NBS1}$ cDNA as a bait in a yeast two-hybrid screening and report the identification of the *A. nidulans* Mre11 homologue (*mreA*). Here, we show the molecular characterization of *sldI*, the *A. nidulans* Rad50 homologue. Interestingly, this gene was identified by the complementation of a synthetic lethal mutation that significantly reduced growth in the absence of cytoplasmic dynein but had little effect on its own (Efimov and Morris, 1998). The $sldI^{RAD50}$ inactivation mutant is sensitive to several DNA-damaging agents and has a deficiency in the DNA replication checkpoint responses. Furthermore, $sldI^{RAD50}$ and $bimE^{APC1}$, the homologue of an anaphase-promoting complex subunit APC1, showed epistatic interactions at S-phase checkpoints and response to hydroxyurea (HU) and UV light.

Results

Identification of the A. nidulans sldI^{RAD50} *gene*

Cytoplasmic dynein is a ubiquitously expressed microtubule motor involved in vesicle transport, mitosis, nuclear migration and spindle orientation (Vallee *et al*., 2004). In the filamentous fungus *A. nidulans*, inactivation of cytoplasmic dynein, although not lethal, severely impairs nuclear migration and reduces colony size (Xiang *et al*., 1994). To investigate the complete range of dynein function, Efimov and Morris (1998) searched for mutations that significantly reduced growth in the absence of dynein but had little effect on their own. They have isolated nine different genes and here we show the identification of one of these genes, *sldI*. Mutation in the *sldI* gene confers absence of conidiation and smaller colony size (Efimov and Morris, 1998). We cloned *sldI* by complementation of conidiation defect and restoration of the normal colony size. The *sldI* gene was cloned by an '*in vitro* ligation' method (Efimov and Morris, 1998). The *sldI1444D* mutant was transformed with a ligation mixture containing *Bam*HI-digested and dephosphorylated vector pAid (that contains the self-replicating fragment AMA1, Gems *et al*., 1991; and *pyrG* as a marker) and genomic DNA fragments from strain R153 obtained by partial digestion with *Sau*3AI, and plated on YAG. Three independent transformants that are prototrophs and dis-

play normal conidiation and no growth defects were obtained. The plasmids that convey this phenotype were rescued by transforming *Escherichia coli* and showed inserts of approximately 5.0 kb (pi1-1), 20.0 kb (pi4-5) and 6.0 kb (pi6-4) respectively. All three inserts contain overlapping fragments and correspond to the same genome region (data not shown). The insert from the plasmid pi1-1 was used for sequencing and further definition of the complementing region. Four subclones from this insert were sequenced and introduced in the *sldI1444D* mutant. Sequencing analysis showed that this insert corresponds to a gene that encodes a protein with high similarity to Rad50 (data not shown; open reading frame AN3619.2; http://www-genome.wi.mit.edu/annotation/fungi/aspergillus/). Only piSK-3 and piSR-1 subclones were able to repair the defect of the *sldI1444D* mutant by integration in the *sldI*^{RAD50} mutated gene (Fig. 1A), suggesting the mutation in the mutated *sldI*^{RAD50} gene is in the central region of the gene. Sequencing analysis of the mutated *sldI*^{RAD50} gene polymerase chain reaction (PCR)-amplified in the *sldI1444D* mutant strain showed a transversion G to C at the nucleotide position 2074 from the open reading frame that corresponds to a novel mutation from Ala to Pro (amino acid 692). This mutation is located into the exon 5 in the hinge *CXXC* domain from Rad50 (Fig. 1B).

The *sldI*^{RAD50} coding region is 4454 nucleotides long, encoding a predicted translation product of 1319 amino acids long with a calculated molecular weight of about 152 KDa and a calculated isoelectric point of 5.54. The *sldI*^{RAD50} has five introns of 230, 101, 51, 48 and 59 bp, located at nucleotide positions 52–282, 285–386, 508–559, 644–692 and 4386–4454 respectively (data not shown). Searches of the *A. nidulans* genome database (http://www-genome.wi.mit.edu/annotation/fungi/aspergillus/) indicated that *sldI*^{RAD50} is a single-copy gene in the *A. nidulans* genome (data not shown). The predicted *sldI*^{RAD50} protein product showed identity with Rad50 of different species (data not shown). The Walker A (WA) and B (WB), and ABC signature motifs from the ABC-type ATPase are located at amino acid positions 51–58, 1241–1251 and 1213–1222 respectively (Fig. 1B). The Q and H loops are located at amino acid positions 179–174 and 1277–1282. The domain of interaction and binding to the Mre11 protein is located at amino acid positions 193–214 (Fig. 1B). The two *coiled-coil* regions and *hinge CXXC* are located at amino acid positions 178–690, 707–1212 and 691–706 respectively (Fig. 1B).

An sldI^{RAD50} *inactivation mutant strain is impaired in the DNA damage response*

To investigate the function of the *sldI*^{RAD50} gene, an *A. nidulans* strain carrying an inactivated *sldI*^{RAD50} gene was generated (Fig. 1C and D; strain IM25). To construct this inactivated strain (IM25), we have deleted the 3′-end of the gene that comprises the final portion of the *coiled-coil* domain and part of the Rad50 region that interacts with Mre11 (see Fig. 1B). We were not able to detect the presence of a *sldI*^{RAD50} mRNA transcript in the IM25 strain by Northern blot and 'real-time' experiments (data not shown). In contrast to the *mreA*^{MRE11} and *scaA*^{NBS1} inactivation mutants (Semighini *et al*., 2003), the *sldI*^{RAD50} inactivated strain did not display striking, abnormal DNA morphology that consists of fragmented nuclei that became highly stretched as hyphal growth continued (data not shown). The IM25 and *sldI1444D* mutant strains have growth differences that probably result from their different genetic backgrounds (Fig. 2). In the absence of any drug, strain *sldI1444D* (derived from XX61 strain) grows poorly while IM25 strain (derived from UI224 strain) grows like the wild-type strain. Strain IM25 was more sensitive than the wild-type strain to several DNA-damaging agents, such as 4-NQO (4-nitroquinoline oxide), CPT (camptothecin), MMS (methyl methane sulphonate) and BLEO (bleomycin) (Fig. 2, second column). Interestingly, the A692P mutation also conferred sensitivity to DNA-damaging agents to the same extent than the inactivation strain, except for HU (Fig. 2, third column). We have also observed that the *sldI*^{RAD50} inactivation strain has increased sensitivity to UV light, both as quiescent or germinating conidia (Fig. 3). The *sldI*^{RAD50} mutant was significantly different from the wild type in the quiescent conidia at 0.22 and 0.25 J m^{-2} s^{-1} ($P < 0.001$) while in the germinating conidia, the *sldI*^{RAD50} mutant was significantly different from the wild type in all UV light intensities ($P < 0.001$).

The A. nidulans sldI^{RAD50} *gene is involved in the S-phase checkpoints*

The *sldI*^{RAD50} inactivation strain is sensitive to replicative stress induced by the drug HU (Fig. 2). HU is an inhibitor of ribonucleoside diphosphate reductase, the rate-limiting step enzyme in deoxyribonucleotide (dNTP) biosynthesis. Depletion of dNTPs activates the DNA replication checkpoint, which slows progression through S-phase (Desany *et al*., 1998). Furthermore, initiation of DNA replication in the presence of high levels of HU causes DNA DSBs (Merrill and Holm, 1999). As HU is an effective inhibitor of DNA synthesis in *A. nidulans* (Bergen and Morris, 1983), we verified whether the *sldI*^{RAD50} could play a role in the S-phase checkpoints by examining whether this mutant could survive a transient period of growth in the presence of HU. Two different assays were used to verify whether DNA replication checkpoint response is impaired in mutant strains (Fagundes *et al*., 2004). The first assay (i.e. the mitosis

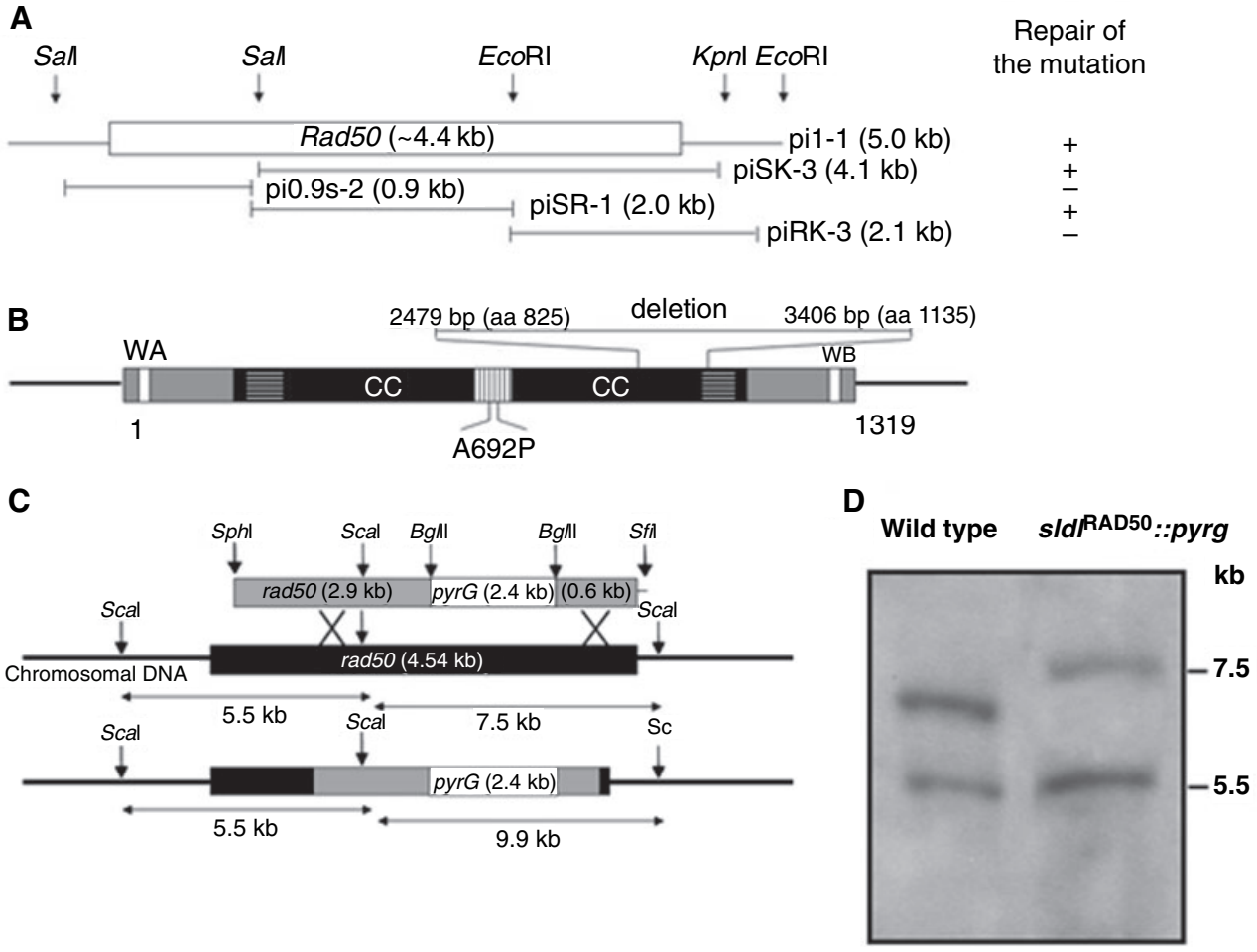

Fig. 1. Complementation of the *sldI1444D* mutant and construction of a *sldI*^{RAD50} inactivation strain.
A. Subclones of the 5.0 kb DNA fragment (pi1-1) that complemented the poor conidiation and growth defects in *A. nidulans sldI1444D* mutant were transformed in this mutant aiming to define the complementing region. Positive and negative signals indicate complementation and absence of complementation respectively.
B. Organization of the domains of the *A. nidulans* SldI^{RAD50} (WA, Walker A; WB, Walker B; Mre11-binding domains are represented by horizontal hatched boxes and the hinge *CXXC* domain is represented by a vertical hatched box; *coiled-coil* regions are shown in the filled boxes). The scheme also shows the region from amino acids 825 to 1135 that was deleted in the IM25 mutant. The region where the mutation is located is also indicated (A692P).
C. The *sldI*^{RAD50} gene was PCR-amplified and ligated to the vector pEYFP, originating pEYFP-*rad50*. A fragment of 924 bp was released from pEYFP-*rad50* and the *zeo-pyrG* cassette that has the *zeo* gene that confers resistance to zeocin and the *pyrG* gene from *A. fumigatus* that confers prototrophy to uracil and uridine was PCR-amplified and ligated in the linearized pEYFP-*rad50*. This new vector was digested with the enzymes *Sfi*I and *Sph*I and a linear fragment of approximately 7.0 kb containing flanking regions of the *sldI*^{RAD50} plus the *zeo-pyrG* cassette was used to transform the strain UI224 to uracil and uridine prototrophy.
D. Southern analysis of the IM25 (*sldI::pyrG*) and wild-type strains. DNA from IM25 and wild-type strains was isolated and cleaved with enzyme *Sca*I; *sldI* gene was used as hybridization probe.

assay) monitors mitosis in mutant and wild-type strains incubated in 6 mM or 100 mM of HU for 5–7 h. The number of nuclei was assessed by 4′,6-diamino-2-phenyylindole (DAPI) staining, and if germlings had two or more nuclei after the HU incubation, they were scored as defective in mitosis arrest (Table 1). The second assay (i.e. the viability assay) assesses germling viability after incubation for 6 h in the presence or absence of 6 mM or 100 mM of HU (Table 2). Both assays measure the state of the replication checkpoint response. The *sldI*^{RAD50} inactivation strain showed to have defects in both mitosis and viability assays at 6 mM and 100 mM (Tables 1 and 2).

A hallmark of the Mre11 complex inactivation phenotype is the radioresistant DNA synthesis (RDS; for a review, see D'Amours and Jackson, 2002). As a first step to verify whether the *A. nidulans sldI*^{RAD50} gene is involved in RDS, we investigated whether the *A. nidulans sldI*^{RAD50} inactivation strain germlings could slow their nuclei division in the presence of the radiomimetic drug bleomycin

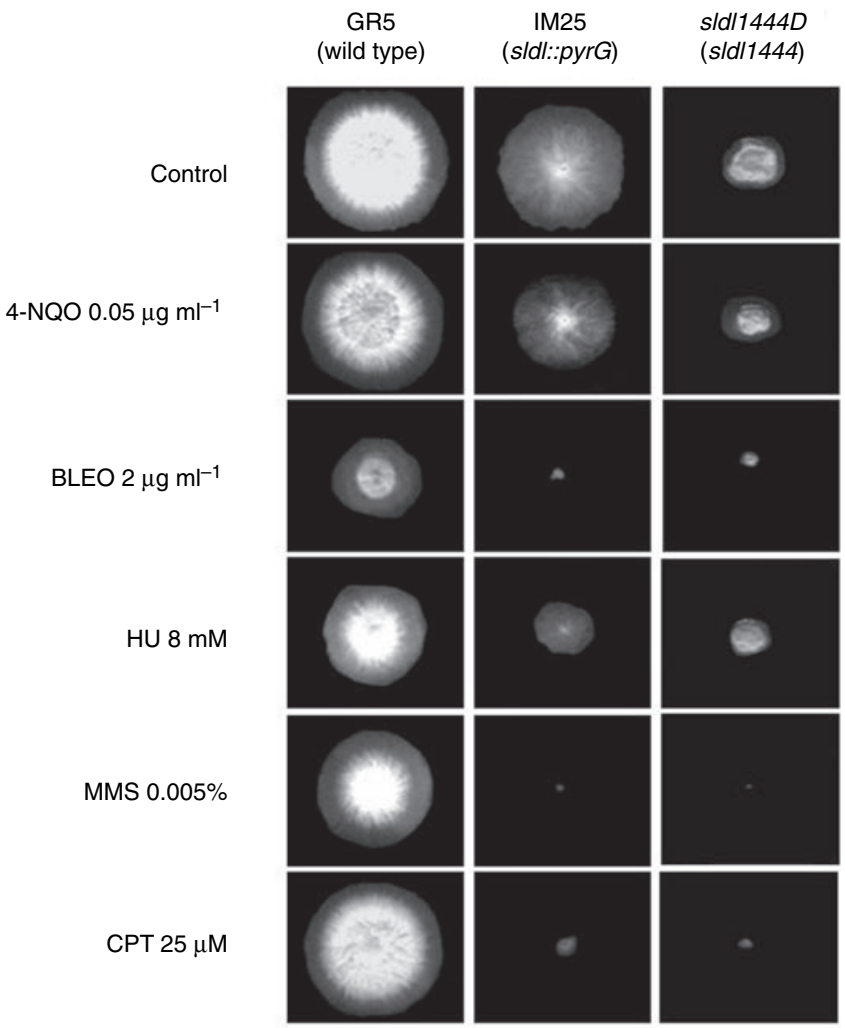

Fig. 2. Growth phenotypes of the GR5 (wild type), IM25 (*sldI::pyrG*) and *sldI1444D* (*sldI1)*. The strains were grown for 72 h at 37°C in YUU medium in the presence or absence of 4-NQO, BLEO, HU, MMS and CPT.

(BLEO). As it can be seen in the Fig. 4, the *sldI1444D* (*sldI1*) mutant and *sldI*^{RAD50} inactivation (IM25) strains showed no growth delay after BLEO release suggesting this strain could display the RDS phenotype. The same behaviour is observed for the *mreA*^{MRE11} inactivation strain (TMRE). Interestingly, the *sldI1444D* (*sldI1*) mutant and *sldI*^{RAD50} inactivation (IM25) strains showed a faster nuclear division than the wild-type and *mreA*^{MRE11} inactivation strain (Fig. 4B and C). The wild-type strain does show growth delay in the presence of BLEO, indicating this strain has an intact checkpoint upon DNA damage caused by BLEO. These data implied that there is no delay of the S-phase when germlings of both *sldI*^{RAD50} and *mreA*^{MRE11} inactivation strains are exposed to the DNA damage caused by BLEO.

Epistasis grouping of A. nidulans scaA^{NBS1}, bimE^{APC1} *and* uvsB^{ATR}

We have shown that *scaA*^{NBS1} and *uvsB*^{ATR} genes are

Table 1. Mitosis assay from *A. nidulans* wild-type and mutant strains.[a]

HU	0 mM	6 mM	100 mM
GR5 (wild type)	63.0 ± 12.7	8.5 ± 0.7	1.5 ± 0.7
T20 (*scaA::pyr-4*)	69.3 ± 2.1	39.0 ± 6.0[b]	8.5 ± 2.6[b]
IM25 (*sldI::pyrG*)	61.0 ± 2.1	23.0 ± 3.1[b]	3.0 ± 0.7[b]
A776 (*bimE7*)	39.3 ± 2.5	23.3 ± 5.9[b]	0 ± 0
IMT (*sldI::pyrG scaA::pyr-4*)	66.5 ± 3.7	22.3 ± 2.2[b]	2.0 ± 0.8
IM238 (*sldI::pyrG bimE7*)	66.5 ± 4.2	26.3 ± 3.0[b]	6.5 ± 3.5[b]
IM14 (*sldI::pyrG ΔuvsB*)	60.5 ± 7.7	25.0 ± 2.8[b]	1.0 ± 0

a. Percentages of germlings that had two or more nuclei after the HU incubation were scored as germlings that did not have mitosis arrest. All the results are the average of determinations from three independent experiments with 100 germlings being evaluated in each. Germlings that did not have mitosis arrest were scored. The results were expressed as mean ± standard deviation. Statistical differences were determined by one-way analysis of variance (ANOVA) followed, when significant, by Newman–Keuls multiple comparison test, using Sigma Stat statistical software (Jandel Scientifics). $P < 0.05$ was considered statistically significant.
b. Significantly different from the wild type.

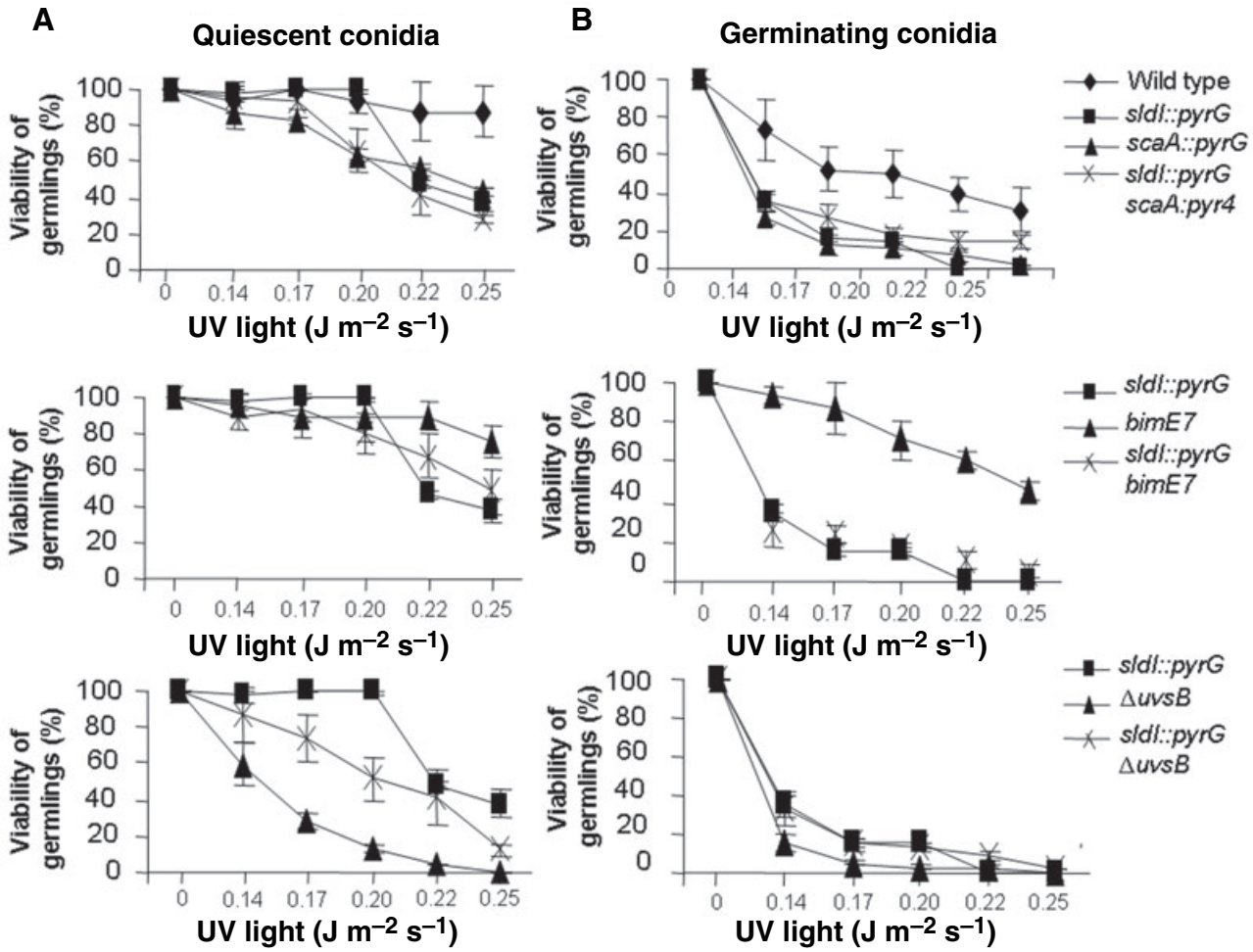

Fig. 3. *sldI*^{RAD50} and *bimE*^{APC1} are interacting in monitoring and/or repairing UV light sensitivity in quiescent conidia. Quiescent (A) and germinating (B) conidiospores from different strains were exposed to UV light, and viability was scored after exposure to this DNA-damaging agent. Viability was determined as the percentage of colonies on treated plates compared with untreated controls. The results were expressed by the average of four independent experiments and means ±standard deviation. Statistical differences were determined by one-way analysis of variance (ANOVA) followed, when significant, by Newman–Keuls multiple comparison test, using Sigma Stat statistical software (Jandel Scientific).

Table 2. Viability assay from *A. nidulans* wild-type and mutant strains.[a]

HU	6 mM	100 mM
GR5 (wild type)	100.0 ± 0	100.0 ± 0
T20 (*scaA::pyr-4*)	78.2 ± 8.1[b]	70.0 ± 6.3[b]
IM25 (*sldI::pyrG*)	78.2 ± 2.8[b]	54.2 ± 2.4[b]
A776 (*bimE7*)	94.8 ± 6.5	97.8 ± 4.4
IMT (*sldI::pyrG scaA::pyr-4*)	78.9 ± 11.9[b]	78.5 ± 11.7[b]
IM238 (*sldI::pyrG bimE7*)	46.3 ± 6.4[b]	37.5 ± 4.0[b]
IM14 (*sldI::pyrG ΔuvsB*)	59.8 ± 3.3[b]	vv67.5 ± 5.1[b]

a. Viability was determined as the percentage of colonies on HU-treated plates compared with untreated controls. All the results are the average of determinations from three independent experiments. The data are the average of three repetitions and means ± standard deviation are shown. Statistical differences were determined by one-way analysis of variance (ANOVA) followed, when significant, by Newman–Keuls multiple comparison test, using Sigma Stat statistical software (Jandel Scientific). $P < 0.05$ was considered statistically significant.
b. Significantly different from the wild type.

involved in S-phase checkpoint (Semighini *et al*., 2003; Fagundes *et al*., 2004). Ye *et al*. (1996) have shown the existence of two S-phase checkpoint regulatory systems that control initiation in mitosis. One that involves both Y15 phosphorylation of p34^{Cdc2} and BimE^{APC1} prevents initiation of mitosis when DNA replication is arrested, and the other that restrains mitosis via Y15 phosphorylation of p34^{Cdc2} when DNA replication is slowed. Thus, aiming to investigate possible genetic interactions among *sldI*^{RAD50} and *scaA*^{NBS1}, *bimE*^{APC1} and *uvsB*^{ATR} during recovery from replication stress and DNA damage, we constructed double mutants *sldI*^{RAD50}*::pyrG*, *scaA*^{NBS1}*::pyr4*, *sldI*^{RAD50}*::pyrG scaA*^{NBS1}*::pyr4* and *sldI*^{RAD50}*::pyrG ΔuvsB*^{ATR}. Epistasis grouping of these mutants was determined by comparison of the mutagen/HU sensitivity of the double repair-deficient mutant with that of the parental simple mutants. When a double mutant was highly sensitive to a mutagen/

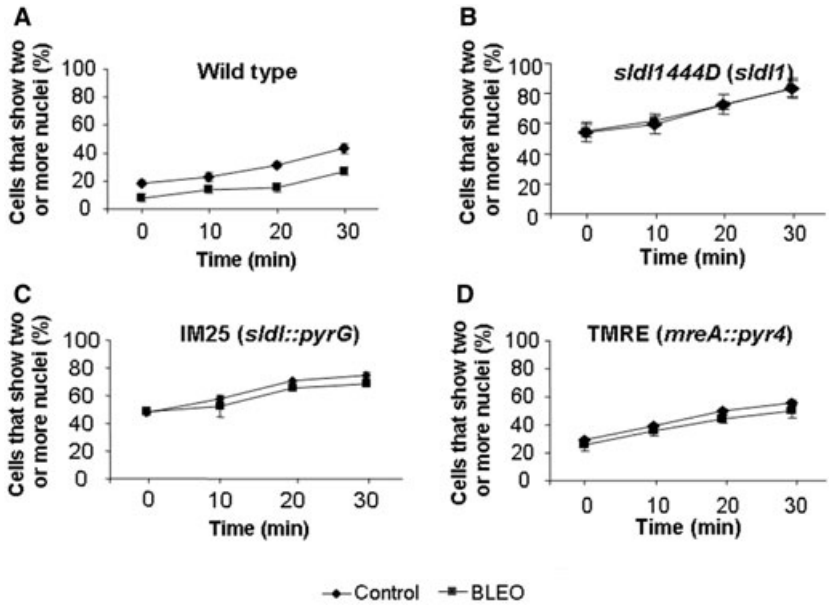

Fig. 4. Radioresistant (RDS) phenotypes of the GR5 (wild type), IM25 (*sldI::pyrG*), *sldI1444D* (*sldI1444*) and TMRE (*mreA::pyr4*). For the growth in the presence of BLEO, conidiospores from GR5, IM25, *sldI1444D* and TMRE strains were first inoculated onto coverslips in YG medium (plus uridine and uracil if necessary), and incubated at 37°C for 5 h. After this period of growth, germlings were exposed to 1 μg ml^{-1} of BLEO for 30 min, then released into drug-free media and samples were taken at 10 min intervals. Control treatments without BLEO were performed for each strain and the number of nuclei was assessed by DAPI staining.

HU, compared with the two parental strains, the relation of the two DNA repair mutations was judged as 'synergistic', suggesting that the two DNA repair genes were functioning in different repair pathways. On the other hand, when the double mutant showed sensitivity similar to either of the parental strains, it was judged as 'epistatic', suggesting that both repair genes were working in the same repair pathway.

The double mutant strain *sldI*^{RAD50}*::pyrG bimE7* was more sensitive to HU than the corresponding parental strains (Fig. 5). The double mutant *sldI*^{RAD50}*::pyrG scaA::pyr4* was inhibited to the same extent as the corresponding parental strains when grown in the presence of 4-NQO, HU, CPT, but not MMS (data not shown). Our results suggest that *sldI*^{RAD50} *bimE*^{APC1} genes showed synergistic interaction while the double mutant *sldI*^{RAD50}*::pyrG scaA::pyr4* is viable and displays the expected epistatic interaction.

We also verified whether the DNA replication checkpoint response was intact in these double mutant strains (Tables 1 and 2). The *bimE7* mutant has an impaired mitosis assay at 6 mM and intact at 100 mM, but remarkably the viability assay is intact at both 6 and 100 mM (Tables 1 and 2). During the mitosis assay at 6 mM, it was observed that an epistatic interaction among *sldI*^{RAD50} and *bimE*^{APC1} and the *sldI*^{RAD50} and *scaA*^{NBS1} ($P > 0.05$) as the double mutants were no worse than either of the parents (Table 1). At 100 mM, there is also an epistatic interaction between *sldI*^{RAD50} and *bimE*^{APC1} (Table 1). Again, in the viability assay at 6 and 100 mM there is a synergistic interaction between *sldI*^{RAD50} and *bimE*^{APC1} (Table 2). As expected, the genes *sldI*^{RAD50} and *scaA*^{NBS1} showed epistasis in the viability assay at both 6 and 100 mM (Table 2). These results strongly suggest that there are epistatic interactions between *sldI*^{RAD50} and *bimE*^{APC1} during DNA replication checkpoint response.

As the UV light interferes with the function of replication forks (Nyberg *et al*., 2002), we checked UV sensitivities of non-dividing (quiescent) and dividing (germinating) double mutants aiming to verify possible interactions between these genes. As expected, when UV irradiation was applied to quiescent and germinating conidia of the double mutant *sldI::pyrG scaA::pyr4*, there is an epistasis between *sldI*^{RAD50} and *scaA*^{NBS1} (Fig. 3A and B, first panels; *P*-value > 0.05). We also observed an epistasis when UV irradiation was applied to quiescent conidia of the *sldI::pyrG bimE7* double mutant (Fig. 3A, second panel left; *P*-value > 0.05). These results indicate once more that *sldI*^{RAD50} and *bimE*^{APC1} have epistatic interactions. Interestingly, when UV irradiation was applied to quiescent conidia, the loss of *sldI*^{RAD50} can partially suppress the *ΔuvsB*^{ATR} UV sensitivity (Fig. 3A, third panel left; *P*-value < 0.005). In addition, when germinating conidia were exposed to UV light, the *sldI::pyrG ΔuvsB*^{ATR} double mutant strain was as sensitive to UV irradiation as either single mutant strains, *sldI::pyrG* or *ΔuvsB*^{ATR} (Fig. 3B, third panel right; $P > 0.05$). In the latter experiment, conidiospores were first allowed to germinate for 4.5 h before UV irradiation was applied, by which time they had entered the first cell cycle. These results show that *sldI*^{RAD50} and *ΔuvsB*^{ATR} are epistatic to monitor and/or repair UV light sensitivity when conidiospores are germinating. We are currently investigating other possible interactions between the Mre11 complex and *uvsB*^{ATR} (M.R.Z.K. Fagundes *et al*., in preparation).

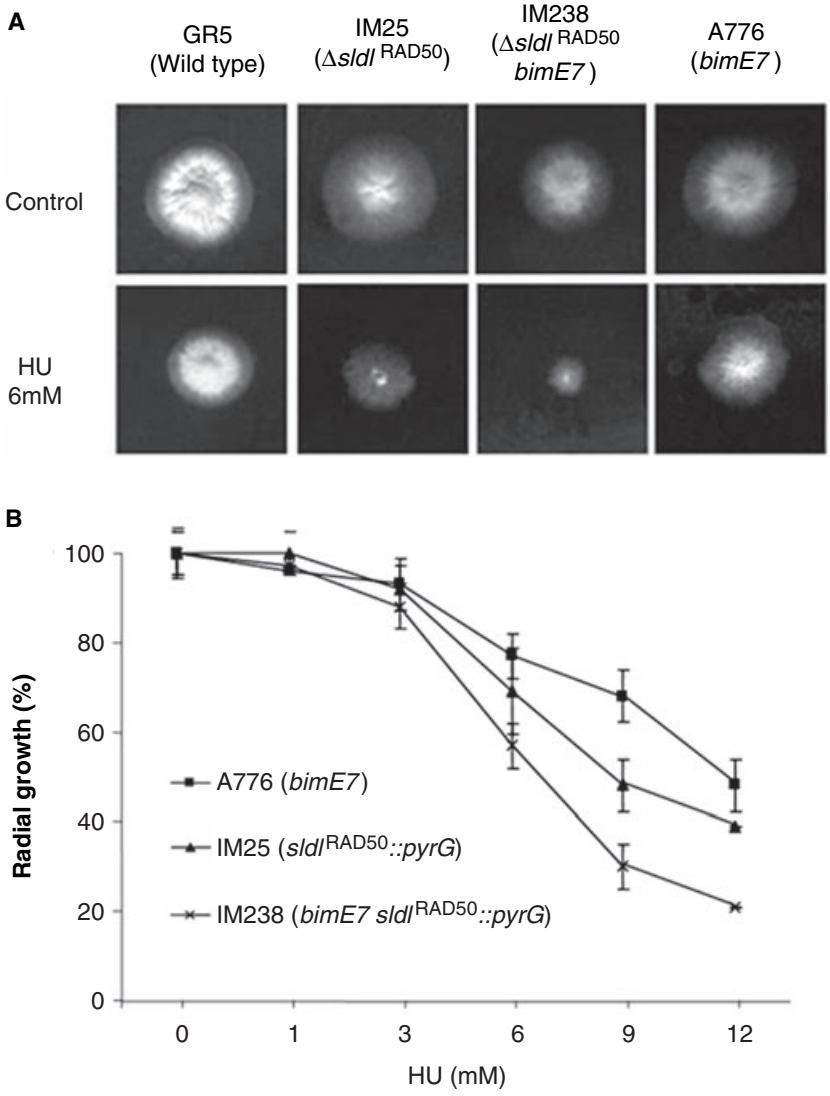

Fig. 5. Growth phenotypes of the GR5 (wild type), IM25 (*sldI::pyrG*), IM238 (*sldI::pyrG bimE7*) and A776 (*bimE7*).
A. The strains were grown for 72 h at 30°C in YUU medium in the presence or absence of HU.
B. Conidia from A776, IM25 and IM238 strains were inoculated and percentage of radial growth inhibition when compared with each strain grown in the absence of HU was evaluated after growth for 72 h at 30°C.

The sldI^{RAD50} *inactivation mutant strain is meiotically defective*

Several species that are deficient in the Mre11 complex cannot successfully execute the meiotic programme, as induction of meiosis leads to lethality (D'Amours and Jackson, 2002). As an initial approach to understand the involvement of the *rad50* gene in the meiotic process in *A. nidulans*, we tested the fertility of the IM25 strain. *A. nidulans* is homothalic, i.e. a single conidium normally has the potential to form hyphae that under special environmental conditions proceed through the sexual cycle. Self-fertilization of either parent may still occur when conidia from two different strains are grown together, but an outcross between the two parents can also take place and will result in the formation of recombinant progeny. In either case, sexual maturity can be visually monitored by the appearance of fruiting bodies (0.1–0.2 mm diameter), called cleistothecia. The sexual spores, ascospores, are enclosed within the cleistothecia. We analysed the ability of IM25 strain to perform self- and cross-fertilization, and determined ascospore yield and viability in the viable cleistothecia (Table 3). When self-fertilized, the wild-type strain showed 100% cleistothecia producing viable ascospores with an average ascospore yield per cleistothecium of $3.3 \pm 0.1 \times 10^5$; 75.6% of these ascospores are viable. The IM25 strain showed 62% cleitothecial viability with about 10 times less ascospores per cleistothecium, but decreased ascospore viability (6.1%). When cross-fertilized, one copy of the inactivated *rad50* is sufficient to retain fertility, the ascospore viability is 66.6%. These results indicate that the *rad50* inactivation is recessive and that *rad50* gene is important for ascospore viability in *A. nidulans*.

Table 3. The *A. nidulans sldI*^{RAD50} is essential for gametogenesis.

	Self-fertilization		Cross-fertilization
	sldI[a] (GR5)	Δ*sldI* (IM25)	*sldI*[b] x Δ*sldI*[c]
Cleistothecia viability[d] (%)	100.0	62.0	100.0
Ascospore yield[e]	$3.3 \pm 0.1 \times 10^5$	$1.6 \pm 1.0 \times 10^4$	$3.4 \pm 2.7 \times 10^4$
Ascospore viability[f]	75.6 ± 5.0	6.3 ± 3.0	66.6 ± 15.9

a. Strain GR5 (see Table 5).
b. Strain R21.
c. Strain UI224 was used for disruption.
d. Number of cleistothecia with viable ascospores divided by the total number of analysed cleistothecia.
e. Recorded values represent the average number of ascospores per cleistothecium estimates for three cleistothecia. For the self-crosses, the entire contents from more than 50 cleistothecia were examined microscopically for the presence of ascospores.
f. Recorded values represent the average from three cleistothecia of the per cent of ascospores having germ tubes at about 8 h.

The Mre11 complex is defective in homologous recombination

As the *rad50* and *scaA*^{NBS1} mutations are epistatic, we decided to use the *scaA1* mutation to determine whether a defective Mre11 complex could affect the frequency of homologous integration. Thus, we transformed an integrative vector that contains the *pyr4* gene of *Neurospora crassa* and the *scsA*^{TOP1} gene, which encodes *A. nidulans* topoisomerase I (Fagundes *et al*., 2003), into wild-type, *scaA*^{NBS1} and *scsA*^{TOP1} mutant strains. All these strains were *pyrG89* and transformants were selected for prototrophy to uracil and uridine. DNA was isolated from the different transformants and the type of integration was analysed by Southern blot. When compared with the wild-type and *scsA*^{TOP1} mutant strains, the *scaA*^{NBS1} mutant is unable to integrate exogenous DNA via homologous recombination (Table 4). These results indicate homologous recombination is dependent on *scaA*^{NBS1} function in the Mre11 complex.

Discussion

Efimov and Morris (1998) screened for mutations that significantly reduced growth in the absence of dynein but had little effect on their own. Here we report that the *sldI* gene is the *A. nidulans* homologue of the Rad50 gene. Interestingly the *sldI* mutant shows synthetic lethality not only with dynein but also with the mitotic kinesin *bimC* (Efimov and Morris, 1998). In total, six out of nine *sld* genes have been cloned. *sldA* and *sldB* are homologues of spindle assembly checkpoint genes BUB1 and BUB3 respectively (Efimov and Morris, 1998). *sldC* is homologous to *S. cerevisiae DNA2* gene, which is a DNA helicase/nuclease (V.P. Efimov, unpublished). *sldD* is type II DNA topoisomerase (V.P. Efimov, unpublished). *sldE* encodes a novel open reading frame without obvious homologies to characterized proteins (V.P. Efimov, unpublished). We do not know why the screen produced so many genes involved in maintaining DNA integrity. It is possible that the screen was not stringent enough, that the interactions between dynein and *sld* mutants are not strong enough (not synthetically lethal, but synthetically sick) and that *sld* genes have no relationship to the dynein's pathway. Another alternative explanation is that mutations in *sldA*, *-B*, *-C*, *-D* and *-I* each cause cohesion defects that are near lethal in the absence of dynein function. Hartsuiker *et al*. (2001) have shown that *Schizosaccharomyces pombe* Rad50 stimulates sister chromatid recombination and links cohesion with repair. Thus, dynein mutation could cause mitotic defects (Efimov and Morris, 1998) which are intensified by loss of cohesion. A more compelling speculation is that the apparatus for sensing and/or repairing DNA damage relies on motor proteins in order to be positioned in different subnuclear locations aiming to perform their different functions. As dynein is a retrograde motor, it could facilitate transport of DNA repair or checkpoint proteins into nuclei. For instance, the DNA damage-induced nuclear localization of p53 requires the minus end-directed microtubule motor dynein (Giannakakou *et al*., 2000; 2002). Disruption of the dynein's motor activity by microinjection of an antibody against the intermediate chain (IC74) of the motor or overexpressing dynamitin (p50) results in impaired accumulation of p53 in the nucleus following DNA damage (Giannakakou *et al*., 2000). The molecular basis of dynein-mediated p53 nuclear trafficking is poorly under-

Table 4. Frequency of homologous versus ectopic integration of transforming DNA in different strains of *A. nidulans.*

Strains	% of homologous integration	% of ectopic integration
scaA$^+$ *scsA*$^+$ ($n^a = 18$)	27	73
scaA$^-$ *scsA*$^+$ ($n = 12$)	0	100
scaA$^+$ *scsA*$^-$ ($n = 17$)	35	65

a. Number of transformants where the homologous integration of the *scsA*^{TOP1} gene was verified by Southern blot.

stood, as p53 is not known to bind directly to the dynein motor complex. Recently, Lo *et al*. (2004) reported that the 8 kDa light chain (LC8) of dynein binds to p53 binding-protein 1 (53BP1). The LC8-binding domain was mapped to a short peptide segment immediately N-terminal to the kinetochore localization region of 53BP1. Therefore, 53BP1 can potentially act as an adaptor to assemble p53 to the dynein complex. Galigniana *et al*. (2004) have shown that hsp90/immunophilin complex might act as an adaptor to link p53 to a subunit of dynactin within the dynein motor complex in human colon cancer cells. During meiosis in fission yeast the nucleus undergoes dramatic dynein-powered movements that are required for efficient homologous chromosome recombination (Yamamoto *et al*., 1999). In *S. cerevisiae* microtubule-dependent nuclear oscillations can facilitate chromosome breakage and recombination (Thrower *et al*., 2003). We are currently constructing Rad50::gfp fusions aiming to verify whether the Rad50 positioning into the nucleus upon DNA damage is dependent on dynein and kinesin mutant backgrounds (I. Malavazi, unpublished results).

Rad50 is part of the heterotetrameric $(Mre11)_2/(Rad50)_2$ complex (Hopfner *et al*., 2001). In higher eukaryotes, null mutations in components of the Mre11 complex are lethal (Luo *et al*., 1999; Yamaguchi-Iwai *et al*., 1999; Zhu *et al*., 2001; Gorski *et al*., 2004), while hypomorphic mutations in NBS1 and MRE11 cause the genome instability syndromes NBS and Ataxia telangiectasia-like disease (ATLD) respectively (Carney *et al*., 1998; Matsuura *et al*., 1998; Varon *et al*., 1998; Stewart *et al*., 1999). A hypomorphic mutation in RAD50, originally identified as a separation of function mutation (Rad50S) in yeast (Alani *et al*., 1990), results in partial embryonic lethality and cancer susceptibility in mice (Bender *et al*., 2002). *S. cerevisiae Rad50S* mutants are blocked early in meiotic progression (Keeney *et al*., 1997). The *A. nidulans sldI1* mutation is present in the hinge *CXXC* domain and sldI1444D (*sldI1*) and *sldI*^{RAD50}*::pyrG* mutant strains have shown the same degree of susceptibility to DNA-damaging agents (except HU) and disruption in the cell cycle checkpoint into the S-phase upon exposure to the mimetic agent BLEO. Two Mre11 complexes bound to DSB ends interact through *CXXC* zink-hook motifs located in the *coiled-coil* region of the Rad50 (Hopfner *et al*., 2002a). The *CXXC* motif promotes a metal-dependent interaction between Rad50 molecules *in vivo* and *in vitro* and consequently dimerization (Hopfner *et al*., 2002a). The *CXXC* motif forms a molecular hook assembly at the apex of the Rad50 *coiled-coil.* This two-cysteine hook creates one-half of a composite Zn^{+2} binding site that links two Rad50-*CXXC* molecules (Hopfner *et al*., 2002a). The Mre11 complex binds to DNA ends through the Mre11 protein, with the coiled-coil arms of Rad50 extending outwards and interacting with an Mre11 complex on the other side of the break through their *CXXC* motifs (Van den Bosch *et al*., 2003). The Zn-hook is critical in prevention of chromosome damage (Lobachev *et al*., 2004). Possibly, the observed *A. nidulans sldI1* mutation in the *CXXC* motif does not allow the formation of the Rad50 dimer and the approximation of the two DNA end breaks, and consequently the absence of Mre11 complex formation.

The Mre11 complex is a key component in the cellular responses to DSBs and is involved in virtually all aspects of DNA end metabolism, including DSB detection, DSB processing, homologous recombination and meiosis, NHEJ, telomere maintenance and the DSB-activated cell cycle checkpoint response (for reviews, see D'Amours and Jackson, 2002; Hopfner *et al*., 2002a; Van den Bosch *et al*., 2003; Assenmacher and Hopfner, 2004). This great number of functions can be explained by the fact that the Mre11 complex combines several enzymatic and non-enzymatic activities, including DNA end processing, DNA cohesion and activation of the damage checkpoint kinases ATM, and possibly ATR (Assenmacher and Hopfner, 2004; Nakada *et al*., 2004). Here, we evaluated the following aspects of the *sldI*^{RAD50} gene in the Mre11 complex: (i) sensitivity to DNA-damaging agents, (ii) the S-phase cell cycle checkpoint response to replicational stress, (iii) epistasis grouping and (iv) homologous recombination and meiosis.

We have previously shown that *mreA*^{MRE11} and *scaA*^{NBS1} genes are involved in S-phase checkpoint (Semighini *et al*., 2003). The *sldI*^{RAD50} inactivation strain showed to have defects in both mitosis and viability assays at 6 mM and 100 mM. Furthermore, there is no delay of the S-phase when germlings of both *sldI*^{RAD50} and *mreA*^{MRE11} inactivation strains are exposed to the DNA damage caused by BLEO. There are at least two S-phase checkpoint mechanisms controlling mitosis in *A. nidulans* (Ye *et al*., 1996). The first S-phase checkpoint is activated during DNA replication that has been slowed by addition of HU to a level that does not arrest replication. It responds to the rate of DNA replication and inhibits mitosis via tyrosine phosphorylation of NimX^{Cdc2}. If DNA replication is arrested, a second checkpoint involves BimE^{APC1}, a subunit of the large ubiquitin ligase called the anaphase-promoting complex (APC) or cyclosome. This second S-phase checkpoint occurs when DNA replication is completely inhibited by higher levels of HU than that which stimulates the prolonged DNA replication checkpoint. This information was obtained through studies of double mutants of *A. nidulans* containing *nimX*^{Cdc2AF} [a mutated version in which the Thr14 is converted to an Ala (A) and Tyr15 to a Phe (F) residue] and the temperature-sensitive mutation *bimE7* (Osmani and Ye, 1997). Either *Cdc2*AF or the *bimE7* mutation alone has a limited capacity to promote mitosis when S-phase is arrested, but, in combination, these two defects allow cells to enter

a lethal premature mitosis before completion of DNA replication.

We investigated the possible genetic interactions between SldI^{RAD50} and BimE^{APC1} by constructing a double mutant *sldI::pyrG bimE*^{APC1}. These two genes are genetically interacting based on the following evidences: (i) the double mutant strain was more sensitive to HU than the corresponding parental strains, (ii) during the mitosis assay at 6 and 100 mM the double mutant has shown epistasis to the corresponding parental mutants, (iii) in the viability assay at 6 and 100 mM there is a synergism between *sldI*^{RAD50} and *bimE*^{APC1} and (iv) an epistatic relationship was also observed when UV irradiation was applied to quiescent conidia of the *sldI::pyrG bimE7* double mutant. Taken together these results suggest a possible novel feature of the Mre11 complex in *A. nidulans*, i.e. a relationship with *bimE*^{APC1} At this moment, we can only speculate on the molecular basis for these interactions. It is possible *sldI*^{RAD50} be active in the NimX^{cdc2} pathway for the control of checkpoint responses. Additionally, this interaction could reveal a relationship between DSBs and the mitotic spindle checkpoint. Lobachev *et al*. (2004) have shown that the Mre11 complex is required *in vivo* to hold the two ends of a broken chromosome together throughout DSB repair. The same authors have shown that a mutation in the Rad50 Zn-hook structure resulted in DNA ends that are dispersed in approximately 10–20% of cells. The Mre11 complex holds broken ends together and counteracts mitotic spindle forces that can be destructive to damaged chromosomes (Lobachev *et al*., 2004). During mitosis in undamaged wild-type cells, the sister chromatids are separated after proteolytic cleavage of cohesin by the pulling forces of the centromere-attached mitotic spindle (Uhlmann, 2004). The cohesion of the sister chromatids is established by the cohesion protein complex during DNA replication and persists until chromosome segregation (Haering and Nasmyth, 2003). The spindle checkpoint ensures the high-fidelity transmission by the controlled proteolytic cleavage of a subunit of the cohesion complex, Scc1, by separase, destroying the cohesion between the sister chromatids and triggering the onset of anaphase (for a review, see Yu, 2002). The separase proteolytic activity is blocked by securin, and the APC in association with one of its substrate cofactors tags the securin proteins with polyubiquitin chains (Peters, 2002). Finally, degradation of the ubiquitinated securin activates the separase (Yu, 2002). Thus, it is possible that the observed interaction between the *sldI*^{RAD50} inactivated strain and *bimE7* mutant reflects an interaction between the Mre11 complex and the microtubule-mediated forces through the spindle checkpoint that would affect the transition from DSBs to chromosome breaks.

Chromosome pairing is followed by cross-over recombination between homologous chromosomes that, in collaboration with sister chromatid cohesion, permits the establishment of temporary connections between homologues. These allow them to orient towards the opposite poles of the meiosis I spindle (Moore and Orr-Weaver, 1998). Recombination between homologous chromosomes often initiates at 'hot spots', which are sites at which DSBs are induced enzymatically. During meiosis, the Mre11 complex plays a role in both formation and processing (5′ to 3′ resection) of the regulated DSBs that initiate meiotic recombination in the yeast *S. cerevisiae* (Alani *et al*., 1990; Cao *et al*., 1990; Johzuka and Ogawa, 1995; Nairz and Klein, 1997). *MRE11* mutants are deficient in the formation of meiotic DSBs and the induction of meiotic recombination and thus do not form viable spores (Ajimura *et al*., 1993; Travassoli *et al*., 1995). *MRE11* may promote DSB formation at hot spots by altering the chromatin configuration (Ohta *et al*., 1998).

We have observed that *sldI*^{RAD50} gene is involved in the viability of sexual spores. We have previously shown that *mreA*^{MRE11} and *scaA*^{NBS1} genes were also involved in the viability of *A. nidulans* sexual spores (Semighini *et al*., 2003). The components of the Mre11 complex are known to be essential for gametogenesis (Ajimura *et al*., 1993; Chin and Villeneuve, 2001; Gallego *et al*., 2001; Bundock and Hooykaas, 2002). Chin and Villeneuve (2001) have shown that meiotic recombination in *Caenorhabditis elegans* is profoundly impaired and perhaps eliminated in the absence of *mre11* function, based on an inability to detect chiasmata cytologically or cross-over recombination events genetically. Gallego *et al*. (2001) showed that in *Arabidopsis thaliana rad50* mutant plants show a sterility phenotype that is present only in plants homozygous for the T-DNA insertion. Bundock and Hooykaas (2002) also found that only *A. thaliana mre11* mutant plants homozygous for T-DNA insertion did not produce pollen. Bleuyard *et al*. (2004) have shown that both male and female gametogenesis are defective in the *Arabidopsis* Rad50 mutant and cytological observation of male meiosis indicates that in the absence of AtRad50 protein, homologous chromosomes are unable to synapse. Furthermore, the atrad50 mutation leads to the destruction of chromosomes during meiosis. Gerecke and Zolan (2000) demonstrated that a *Coprinus cinereus mre11-1* mutant shows a delay in fruitbody formation and a reduction in the number of mushrooms formed per dikaryon. The *C. cinereus mre11-1* mutant also has several meiotic defects. In this mutant, pachytene chromatin condensation is disrupted, and although some meiotic cells appear to achieve metaphase I condensation, no further meiotic progression is observed. In addition, the *mre11-1* mutant also fails to undergo proper chromosome synapsis. By analogy to the roles of Mre11p in yeast, *C. cinereus* and *C. elegans* in meiotic recombination, we suggest that a potential

requirement for *A. nidulans* Mre11 complex in meiosis could be downstream to the initiation step, possibly promoting 5′ to 3′ resection of DSBs, to generate a substrate for a subsequent strand invasion step (Ajimura *et al*., 1993; Nairz and Klein, 1997; Usui *et al*., 1998; Chin and Villeneuve, 2001).

The Mre11 complex participates in two pathways of DSB repair: HR and NHEJ (Petrini, 1999; 2000; D'Amours and Jackson, 2002). HR depends on the undamaged sister chromatid, a homologous DNA template, to accurately restore the continuity of the broken sister chromatid. We investigated whether the *A. nidulans* Mre11 complex was involved in HR by observing the frequency of homologous integration of the topoisomerase I gene in a mutant strain (*scaA1*) of one of the *A. nidulans* Mre11 complex genes, *scaA*^{NBS1}. HR was completely abolished for the gene examined in the *scaA1* strain, indicating the Mre11 complex is critical for HR in *A. nidulans*. Tauchi *et al*. (2002) showed that Nbs1 is essential for HR-mediated repair in higher vertebrate cells. When these authors used a site-specific DSB repair assay, they demonstrated a remarkable reduction of HR events following generation of such breaks in Nbs1-disrupted cells. These observations indicate that the main pathway of HR repair in *A. nidulans* requires a functional Mre11 complex.

Experimental procedures

Strains, media methods and statistical analysis

Aspergillus nidulans strains used are described in Table 5. Media were of two basic types. A complete medium with three variants: YAG (2% glucose, 0.5% yeast extract, 2% agar, trace elements), YUU (YAG supplemented with 1.2 g l^{-1} each of uracil and uridine) and liquid YG or YG + UU medium of the same compositions (but without agar). A modified minimal medium (MM: 1% glucose, original high nitrate salts, trace elements, 2% agar, and pH 6.5). Trace elements, vitamins and nitrate salts are described by Kafer (1977; Appendix, available on request from the author). Standard genetic techniques for *A. nidulans* were used for all strain constructions (Kafer, 1977).

For the UV light viability assays, conidiospores (dormant in a quiescent G1 state) were suspended in 0.2% Tween-20 and plated out on YUU plates (approximately 100 conidia per plate). The plates were then irradiated immediately with UV using a UV Stratalinker 1800 (Stratagene) and incubated at 30°C for 48 h to determine UV sensitivity of non-dividing cells. To determine UV survival of dividing cells, conidiospores on YUU plates were first allowed to germinate for 4.5 h in a 30°C incubator for colony formation. By this time the germinated spores had entered the cell cycle and were about to undergo the first mitosis. These germlings were UV irradiated on the plates and then similarly incubated at 30°C for 48 h. Viability was determined as the percentage of colonies on treated plates compared with untreated controls.

Ascospore yield was determined by clearing cleistothecia of Hulle cells and conidia, opening them individually in water, and counting an aliquot of the ascospores obtained from each in a Neubauer counting chamber. Ascospore viability was determined by germinating the ascospores at 37°C for 8 h in YG medium and microscopically checking 200 ascospores from each cleistothecium for the presence of germ tubes (Kirk and Morris, 1991).

To evaluate the differences among the strains used for growth in the presence of DNA-damaging agents, mitosis, viability and UV light assays, we used one-way ANOVA and Student's Newman Keuls *post hoc* test. The data shown are the average of three independent experiments and means ± standard deviation are shown in Tables 1 and 2 and Figures 3 and 4. Analyses were performed using the software package Sigma Stat (Jandel Scientifics, USA) and the statistical significance was set at $\alpha = 0.05$. In the epistasis grouping, *P*-values > 0.05 and < 0.05 were used to discriminate between epistatic and synergistic interactions respectively.

Inactivation of the A. nidulans *rad50 gene*

The *sldI*^{RAD50} gene was PCR-amplified by using Taq Platinum High Fidelity™ (Invitrogen, USA) with the primers (underlined

Table 5. *Aspergillus nidulans* strains.

Strains	Genotypes	References
GR5	*pyrG89; wA3; pyroA4*	FGSC A773
R153	*wA3; pyroA4*	FGSC
R21	*yA2 pabaA1*	FGSC
UI224	*pyrG89; yA2; argB2*	Semighini *et al*. (2003)
XX61	*pyrG89; pyroA4; wA2; alcA(p)::nudA::pyr4*	Efimov and Morris (1998)
AAH14	*pyrG89 pabaA1; yA2; ΔuvsB*	Hofmann and Harris (2000)
sldI1444D	*pyrG89 yA2; sldI1*	Efimov and Morris (1998)
TMRE	*pyrG89 yA2; argB2; mreA::pyr4*	Semighini *et al*. (2003)
T20	*wA3; pyroA4; scaA::pyr4*	Bruschi *et al*. (2001)
T20-17	*pyrG89; wA3; pyroA1; scaA::pyr4*	Bruschi *et al*. (2001)
IM25	*yA2; argB2; sldI::pyrG*	This work
A776	*pabaA1; acrA1; bimE7; riboB2 chaA1*	FGSC
scs528-180	*pyrG89 pabaA1 yA2; scsA1*	Fagundes *et al*. (2003)
IMT	*pyrG89 yA2; sldI::pyrG; scaA::pyr4*	This work
IM238	*bimE7; sldI::pyrG*	This work
IM14	*pyrG89 yA2; argB2; sldI::pyrG; ΔuvsB*	This work

regions, *Bam*HI sites): RAD50-ST-BHI 5′-CGGGATCCATG CACAGTATTGTTGCC-3′ and RAD50-BHI-3′ 5′-CGGGATC CCATAACCTAGATTAGGTAGTG-3′. The DNA fragment was digested with *Bam*HI and ligated to the vector pEYFP (kindly provided by Dr A.F. Ram) previously digested with *Bam*HI and dephosphorylated by using calf intestine phosphatase (CIP; Invitrogen, USA). The originated vector, pEYFP-*rad50*, was digested with the enzyme *Bgl*II. A fragment of 924 bp was released from pEYFP-*rad50* and the resulting plasmid was dephosphorylated with CIP. The *zeo-pyrG* cassette that has the *zeo* gene that confers resistance to zeocin and the *pyrG* gene from *Aspergillus fumigatus* that confers prototrophy to uracil and uridine was PCR-amplified from vector pCDA21 (a fragment of 2417 bp; Chaveroche *et al*., 2000) by Taq Platinum High Fidelity™ (Invitrogen, USA) using the primers ZEO Bgl II F-5′-TCAGTCCTGCTCGG-3′ e PYR Bgl II R-5′-GCC TCAAACAATGCTCTTC-3′. These primers contain *Bgl*II restriction sites and the PCR product was digested with this enzyme and ligated into the vector pEYFP-RAD50, generating the vector pEYFP-*rad50-zeo pyr*. This vector was digested with the enzymes *Sfi*I and *Sph*I and a linear fragment of approximately 7.0 kb containing the flanking regions of the *sldI*^{RAD50} plus the *zeo-pyrG* cassette was used to transform the strain UI224 to uracil and uridine prototrophy. The transformants were screened for CPT sensitivity and checked for homologous integration by Southern blot analysis. Transformations were performed as described by Osmani *et al*. (1987).

For the homologous recombination experiments, *scsA*^{TOP1}, which encodes *A. nidulans* topoisomerase I, was cloned in the integrative vector pRG3 that contains the *pyr4* gene of *N. crassa*. This plasmid was transformed into the wild-type, *scsA*$^{-}$ and *scaA*$^{-}$ strains. All strains were *pyrG89*, and transformants were selected for prototrophy on YAG medium (i.e. lacking uracyl and uridine). DNA from all transformants was digested with *Sma*I and analysed by Southern blotting using as a probe a 4.5 kb *Eco*RI fragment containing the whole *scsA*^{TOP1} gene. This fragment was labelled using [α-^{32}P]-dCTP and the kit RTS Rad Prime DNA labelling System (Gibco-BRL).

Methods of replication checkpoint response and growth in the presence of BLEO

For the mitosis assay, conidiospores were inoculated onto coverslips in YUU medium with 0, 6 or 100 mM of HU. After 5–7 h incubation at 30°C, coverslips with adherent germlings were transferred to fixative solution (3.7% formaldehyde, 50 mM sodium phosphate buffer pH 7.0, 0.2% Triton X-100) for 30 min at room temperature. Then, they were briefly rinsed with PBS buffer (140 mM NaCl, 2 mM KCl, 10 mM $NaHPO_4$, 1.8 mM KH_2PO_4, pH 7.4) and incubated for 5 min in a solution with 100 ng ml^{-1} DAPI (Sigma Chemical) and 100 ng ml^{-1} calcofluor (Fluorescent brightener, Sigma Chemical). After incubation with the dyes, they were washed with PBS buffer for 5 min at room temperature and then rinsed in distilled water and mounted on the slides. The material was photographed using a Zeiss epifluorescence microscope. The number of nuclei was assessed by DAPI staining. Germlings that had two or more nuclei after the HU incubation were scored as having a non-functional checkpoint response.

For the viability assay, 1.0×10^8 conidia were inoculated in YUU medium with 0, 6 or 100 mM of HU, and incubated in a reciprocal shaker (250 r.p.m.) at 30°C for 7 h. The conidiospores were washed with water, conveniently diluted and plated on YUU and incubated at 30°C for 48 h. Viability was determined as the percentage of grown colonies on plates with drug-treated conidiospores compared with untreated controls.

For the growth in the presence of BLEO, conidiospores from GR5, *sldI1444D*, IM25 and TMRE strains were first inoculated onto coverslips in YG medium (plus uridine and uracil if necessary), and incubated at 37°C for 5 h. After this period of growth, 1 μg ml^{-1} BLEO was added to the culture medium and incubated at 37°C for additional 30 min. Control treatments without BLEO were performed for each strain. The coverslips with adherent germlings were washed four times with fresh medium and re-incubated in appropriate culture medium for 30 min at 37°. Samples were collected every 10 min until 30 min and the number of nuclei was assessed by DAPI staining.

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