A point mutation in the *Aspergillus nidulans sonB\textsuperscript{Nup98}* nuclear pore complex gene causes conditional DNA damage sensitivity

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ABSTRACT

The nuclear pore complex (NPC) is embedded in the nuclear envelope where it mediates transport between the cytoplasm and nucleus and helps organize nuclear architecture. We previously isolated \textit{sonB}1, a mutation encoding a single amino acid substitution within the \textit{Aspergillus nidulans} SONBn\textsuperscript{Nup98} NPC protein (nucleoporin). Here we demonstrate that this mutation causes marked DNA damage sensitivity at 42\degree. Although SONBn\textsuperscript{Nup98} has roles in the G2 transition, we demonstrate that the G2-DNA damage checkpoint is functional in the \textit{sonB}1 mutant at 42\degree. The MRN complex is comprised of MRE11, RAD50 and NBS1 and functions in checkpoint signaling, DNA repair and telomere maintenance. We find at 42\degree, the DNA damage response defect of \textit{sonB}1 mutants causes synthetic lethality when combined with mutations in \textit{scaA}\textsuperscript{NBS1}, the \textit{A. nidulans} homologue of NBS1. We provide evidence that this synthetic lethality is independent of MRN cell cycle checkpoint functions or MREA\textsuperscript{MRE1} mediated DNA repair functions. We also demonstrate that the single \textit{A. nidulans} histone \textit{H2A} gene contains the C-terminal SQE motif of histone H2AX isoforms and that this motif is required for the DNA damage response. We propose that the \textit{sonB}1 nucleoporin mutation causes a defect in a novel part of the DNA damage response.
INTRODUCTION

The nuclear pore complex (NPC) is an evolutionarily conserved structure made up of multiple copies of ~30 different NPC proteins (nucleoporins) embedded in the nuclear envelope (for review see Tran and Wente 2006; Hetzer et al. 2005). The NPC restricts diffusion of proteins and nucleic acids between the nucleus and cytoplasm and facilitates active nucleocytoplasmic transport through the nuclear envelope. Other roles for the NPC are only just beginning to be understood. For example, in *S. cerevisiae* the NPC has been demonstrated to play roles in tethering telomeres to the nuclear periphery which helps facilitate transcriptional silencing of subtelomeric genes (Galy et al. 2000; Feuerbach et al. 2002; Therizols et al. 2006). Somewhat paradoxically, certain nucleoporins have been demonstrated to preferentially associate with transcriptionally active genes (Ishii et al. 2002; Casolari et al. 2004, 2005; Menon et al. 2005; Dilworth et al. 2005; Schmid et al. 2006). Interestingly, budding yeast nucleoporin null alleles have been identified which display sensitivity to DNA damaging agents (Bennett et al. 2001; Chang et al. 2002; Galy et al. 2000; Loiillet et al. 2005; Therizols et al. 2006). Although the mechanism leading to DNA damage sensitivity of these nucleoporin nulls is currently not known, it is likely that NPC function is required for the normal DNA damage response.

In response to DNA damage, cells both activate DNA repair pathways and enforce checkpoints to arrest cell cycle progression until DNA has been repaired (for review see McGowan and Russell 2004; Zhou and Elledge 2000). In the presence of DNA damage, the G2-DNA damage checkpoint prevents mitotic entry via tyrosine phosphorylation of the cyclin dependent kinase Cdc2 (Cdk1 or NimX$^{Cdc2}$ in *A. nidulans*). Tyrosine phosphorylated Cdc2 must be dephosphorylated in order for mitosis to occur and mutation of tyrosine to non-
phosphorylatable phenylalanine (cdc2F mutants) results premature mitotic entry in the presence of DNA damage (Ye et al. 1997; for review see Zhou and Elledge 2000). The evolutionarily conserved phosphatidylinositol 3-kinase-like kinases (PIKK) ATR and ATM function to signal both repair and checkpoint pathways in response to DNA damage. The central importance of ATR and ATM is underscored by human diseases such as ataxia telangiectasia (AT) which result from mutation of these genes. More recently the MRN (MRE11, RAD50, NBS1) complex has been demonstrated to function early in the DNA damage response together with the ATM and ATR kinases and mutations in MRN genes have been linked to the diseases Nijmegen breakage syndrome (NBS) and ataxia telangiectasia-like disorder (ATLD) (Difilippantonio et al. 2005; Jazayeri et al. 2006; Lee and Paull 2005; Stiff et al. 2005; Uziel et al. 2003; You et al. 2005; for review see Abraham and Tibbetts 2005; Stavridi and Halazonetis 2005; Zhang et al. 2006). The MRN complex has roles in cell cycle checkpoint signaling as well as being involved in DNA repair and telomere maintenance (for review see D’Amours and Jackson 2002; Zhang et al. 2006). Mre11p has DNA nuclease, strand dissociation and strand annealing activities, while RAD50 has similarity to structural maintenance of chromosome (SMC) proteins and is thought to form a dimer which bridges DNA strands at a double strand break (for review see D’Amours and Jackson 2002; Stavridi and Halazonetis 2005; Zhang et al. 2006). The precise function of NBS1 is less clear although it contains a forkhead-associated (FHA) and breast cancer C-terminus (BRCT) domain suggesting it binds phosphorylated proteins (Becker et al. 2006; for review see D’Amours and Jackson 2002; Stavridi and Halazonetis 2005; Zhang et al. 2006). Indeed, NBS1 has been demonstrated to bind the γ-H2AX phospho-serine epitope which is phosphorylated early in the DNA damage response by the ATM/ATR kinases in nucleosomes surrounding DNA damage (Downs et al. 2000; Kobayashi et al. 2002; Celeste
Aspergillus nidulans has long been utilized as a model genetic system and the cell cycle and DNA damage response in this organism is well characterized (for review see OSMANI and MIRABITO 2004; OSMANI and Ye 1996; GOLDMAN et al. 2002; GOLDMAN and KAFER, 2004). Temperature sensitive mutants of the A. nidulans nimA kinase reversibly arrest in G2 at the non-permissive temperature of 42\degree even though the Cdc2/CyclinB kinase is fully activated (OSMANI et al. 1987). This is likely because the Cdc2/CyclinB kinase is cytoplasmic at a nimA1 G2 arrest and cannot enter the nucleus (Wu et al. 1998). We have previously isolated mutations in two nucleoporins, SONA\textsuperscript{Gle2} and SONBn\textsuperscript{Nup98}, which suppress a nimA1 G2 arrest and allow entry into mitosis (Wu et al. 1998; De Souza et al. 2003). SONA\textsuperscript{Gle2} and SONBn\textsuperscript{Nup98} both disperse from the NPC during the partial disassembly of the NPC in A. nidulans (De Souza et al. 2004). It is likely that these NPC mutants suppress the nimA1 G2 arrest by allowing sufficient Cdc2/CyclinB and tubulin into the nucleus to allow mitotic entry (Wu et al. 1998; De Souza et al. 2004). Here we show that the sonB1 mutation displays a high degree of sensitivity to DNA damaging agents at 42\degree but that this DNA damage sensitivity is independent of the G2-DNA damage checkpoint. Epistasis analysis indicates that SONBn\textsuperscript{Nup98} functions on a different pathway of the DNA damage response than those involving UVSC\textsuperscript{Rad51}, UVSH\textsuperscript{Rad18}, \gamma-H2AX phosphorylation and the G2-DNA damage checkpoint. The defect in sonB1 mutants that causes DNA damage sensitivity also results in synthetic lethality at 42\degree when combined with mutations in scaA\textsuperscript{NBS1}, which encodes the A. nidulans homologue of NBS1 (BRUSHI et al. 2001; SEMIGHINI et al. 2003). Similar synthetic lethality was not observed between sonB1 and mreA\textsuperscript{MRE11} mutants suggesting that sonB1 synthetic lethality with scaA\textsuperscript{NBS1} mutants is independent of the DNA repair activities of the MRN complex. We provide evidence that the synthetic lethality between sonB1 and
sca$^{\text{NBS1}}$ mutants is also independent of the cell cycle checkpoint functions of SCAA$^{\text{NBS1}}$. Our data suggest that the SONBn$^{\text{Nup98}}$ nucleoporin may have a novel role in the DNA damage response.
MATERIALS AND METHODS

General techniques: Media and general techniques for A. nidulans culture, transformation and DAPI staining for chromosome mitotic index were as previously described (OSMANI et al. 1987, 1991, 1994; OAKLEY and OSMANI, 1993; YE et al. 1995; WU et al. 1998). Western analysis was carried out preparing lysates in sample buffer containing 6 M urea as described previously (DE SOUZA et al. 2000). The phospho-Cdc2 (Tyr15) antibody was purchased from Cell Signaling Technology.

DNA damage sensitivity assays: Quiescent conidiospores and germlings were tested for sensitivity to UV irradiation as previously described (YE et al. 1997) using a microprocessor controlled UV cross linker (FBUVXL-1000; Fischer Biotech; 254 nm). Hydroxyurea, DEO, MMS and camptothecin were all purchased from Sigma and added to media at the appropriate concentrations immediately prior to pouring plates. All plates were pre-warmed to 32° or 42° as appropriate prior to inoculation. Entry into mitosis after MMS treatment of either conidiospores or germlings arrested at the G2 nimA5 arrest point was as described previously (YE et al. 1997).

Plasmid constructs: Serine 129 of the A. nidulans histone H2A gene (MAY and MORRIS, 1987) in plasmid pRG3-H2A-H2B (DE SOUZA et al. 2003) was mutated to alanine using the Quickchange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) to generate plasmid pRG3 H2A S129A-H2B. Introduction of the appropriate mutation was confirmed by sequencing.

A. nidulans strains: Genotypes of strains used in this study are listed in Supplemental Table 1.

Although the mreA^{MRE11} disruption strain (TMRE) was previously reported as being sterile (SEMIGHINI et al. 2003), we were able obtain viable progeny in sexual crosses. The H2A S129A mutant was constructed by a two step gene replacement (Ye et al. 1996). GR5 (pyrG89; wA3; pyroA4) and CDS40 (pyrG89; wA2; pyroA4; sonB1) were transformed with plasmid pRG3 H2A...
S129A-H2B and single site integration at the histone \( H2A/H2B \) locus confirmed by Southern blott analysis and PCR using primers external to the region of duplication (Yang et al. 2004). Plasmid loss was selected for with 5-fluoroorotic acid (Osmani et al. 1994) and evictants maintaining the \( H2A \; S129A \) mutation selected for by screening for DNA damage sensitivity. Introduction of the \( H2A \; S129A \) mutation was confirmed by PCR amplification and sequencing of the histone \( H2A \) locus. Double mutant strains generated between nucleoporin mutants and DNA damage response mutants were confirmed by crossing strains back to a wild type strain to recover the single mutant phenotypes. Strains IM69, MKF11, SCA299-16, and TMRE were kind gifts from Dr. Gustavo Goldman (Universidade de São Paulo, São Paulo, Brazil).
RESULTS

The sonB1 NPC mutant is highly DNA-damage sensitive at 42°: The nimA1 temperature sensitive mutation causes cells to arrest in G2 of the cell cycle at the restrictive temperature of 42°. Intriguingly, we previously isolated single point mutations in two essential nuclear pore complex (NPC) proteins, SONAGle2 and SONBNup98, which suppress the nimA1 G2 arrest and allow mitotic entry at 42° (Wu et al. 1998; De Souza et al. 2003). This suggests that the sonA1 and sonB1 NPC mutants are defective in some aspect of G2 regulation at 42°. As loss of G2 checkpoint functions over mitotic entry can cause DNA damage sensitivity, we tested the ability of sonA1 and sonB1 mutants to form a colony in the presence of DNA damaging agents. Wild type and the G2-checkpoint deficient uvsB505ATR and cdc2F mutant strains were used as controls (Ye et al. 1997; De Souza et al. 1999). Strikingly, the sonB1 mutant displayed marked sensitivity to the DNA alkylating agents MMS and DEO at 42° but behaved similar to wild type at 32° (Figure 1A). Similar results were obtained using survival assays which indicated that the sonB1 mutant was more sensitive to DEO at 42° than the uvsB505ATR mutant (Figure 1B). Moreover, this DNA damage sensitivity was specific to the sonB1 mutant as the sonA1 nucleoporin mutant remained viable at 42° in the presence of DEO or MMS (Figure 1A and B). Importantly, the DNA damage sensitivity of sonB1 mutants at 42° was not due to general cellular stress as sonB1 mutants were not sensitive to nocodazole, or camptothecin at 42° (Figure 1A, and data not shown). Thus the sonB1 mutation causes a defect in the DNA damage response specifically at the temperature at which it suppresses the nimA1 G2 arrest. It is particularly intriguing that a single amino acid substitution within an essential NPC protein (De Souza et al. 2003), would cause sensitivity to DNA damaging agents.
In *A. nidulans*, mutants with defective G2 checkpoint functions are sensitive to DNA damage elicited to germlings which have entered the cell cycle but display little change in viability if DNA damage is elicited to quiescent conidiospores (Ye *et al.* 1997; De Souza *et al.* 1999). This is because after DNA damage has occurred, quiescent conidiospores take several hours to enter the cell cycle allowing time for DNA repair before DNA replication and mitotic entry. We therefore compared the UV irradiation sensitivity of *sonB1* germlings and quiescent conidiospores at 42°. The *cdc2AF* mutant which is defective in the G2 checkpoint over mitotic entry (Ye *et al.* 1997) was also tested as a control. The *sonB1* mutant behaved similar to the *cdc2AF* mutant with germlings, but not conidiospores, displaying sensitivity to UV irradiation at 42° (Figure 2). In contrast, the *uvsH304Rad18* DNA repair deficient mutant (Kafar and Mayor, 1986; Yoon *et al.* 1995) was sensitive to UV irradiation elicited at 42° to either conidiospores or germlings. Notably, the UV irradiation sensitivity of *sonB1* germlings was restricted to 42° (Figure 2), similar to the case for *sonB1* sensitivity to MMS and DEO (Figure 1). These data are consistent with the *sonB1* mutant potentially having a defective G2 DNA damage checkpoint at 42°, the temperature at which this mutation suppresses the nimA1 G2 arrest.

**In *sonB1* mutants, Cdc2 undergoes tyrosine phosphorylation in response to DNA damage:** Similar to humans, the G2 DNA damage checkpoint prevents mitotic entry through a pathway leading to tyrosine phosphorylation of Cdc2 in *A. nidulans* (Ye *et al.* 1997). We therefore examined the ability of *sonB1* mutants to tyrosine phosphorylate Cdc2 in response to DNA damage at 42°. Asynchronous wild type or *sonB1* log phase cultures were shifted to 42° for 3 hr, and then either treated or not with MMS for an additional 2 hr at 42°. Lysates were prepared at each stage and immunoblotted with an antibody specific for tyrosine phosphorylated Cdc2. Cdc2 tyrosine phosphorylation increased similarly in either wild type or the *sonB1* mutant in response
to DNA damage (Figure 3A). To ensure that cells were in G2 prior to MMS addition, we also performed this experiment in strains which contained the nimA5 temperature sensitive mutation which arrests cells in G2 at the restrictive temperature of 42°C (OSMANI et al. 1987). At nimA5 arrest, Cdc2 was not tyrosine phosphorylated in the absence of DNA damage, but became tyrosine phosphorylated following MMS addition (Figure 3B), consistent with our previous results (YE et al. 1997). As shown in Figure 3B, sonB1 mutants were able to tyrosine phosphorylate Cdc2 in response to DNA damage elicited at the G2 nimA5 arrest point. This provides strong evidence that the G2 DNA damage checkpoint pathway leading to Cdc2 tyrosine phosphorylation is functional in sonB1 mutants at 42°C. Interestingly, sonB1 mutants displayed a higher level of tyrosine phosphorylated cdc2 compared to wild type strains (Figure 3A and B). As this was observed at 32°C as well as at 42°C, this effect is likely not related to the DNA damage sensitivity of sonB1 mutants at 42°C but may reflect the demonstrated defect of sonB1 mutants in G2/M regulation (DE SOUZA et al. 2003).

While the above data demonstrate that sonB1 mutants are able to tyrosine phosphorylate Cdc2 in response to DNA damage, it is still possible that a just enough Cdc2/CyclinB is able to enter the nucleus, be activated and allow inappropriate mitotic entry. To determine if this is the case, we next examined if sonB1 mutants enter mitosis prematurely if DNA is damaged. To do this we first synchronized cells at the nimA5 G2 arrest point (YE et al. 1997). These G2 arrested cells were either treated or not with MMS to elicit DNA damage. Cells were then released to the nimA5 permissive temperature of 32°C by media exchange and mitotic entry followed by examining cells for condensed DNA at time points after release. As expected, the nimA5 control delayed mitotic entry in the presence of DNA damage while cells also containing the cdc2AF mutation entered mitosis similarly in the presence or absence of DNA damage (Figure 3C). Cells
containing the sonB1 mutation delayed mitotic entry in the presence of DNA damage (Figure 3C), consistent with these cells having an intact G2 DNA damage checkpoint. However, as sonB1 mutants are not DNA damage sensitive at 32° (Figure 1), it is possible that the delay in mitotic entry of the nimA5 sonB1 mutant in these experiments was due to a rapid reactivation of sonB1 upon shifting cultures to 32°. To determine if sonB1 mutants delay mitotic entry if cells are maintained at 42° we germinated wild type or sonB1 conidiospores at 42° in the presence or absence of MMS and followed entry into the first mitosis. Under these conditions, sonB1 mutants delayed entry into mitosis in the presence of DNA damage similar to a wild type strain (Figure 3D). Together, these results indicate that sonB1 mutants have a functional G2 DNA damage checkpoint, even though sonB1 germlings are more sensitive to UV irradiation than are sonB1 quiescent conidiospores.

**sonB1 mutants display synthetic lethality with scaA^{NBS1} mutants at 42° without DNA damage:** The above indicates that the sonB1 mutant is defective in some part of the DNA damage response other than the G2 DNA damage checkpoint. To further investigate this, we determined if sonB1 mutants genetically interacted with mutants defective in different aspects of the DNA damage response in *A. nidulans* (Brushi et al. 2001; De Souza et al. 1999; Hofmann and Harris 2000; Kafer and Mayor 1986; Kafer and May; 1997; Malavazi et al. 2006; Nayak et al. 2006; Semighini et al. 2003; Van Heemst et al. 1997; Ye et al. 1997; Yoon et al. 1995). Most striking were the genetic interactions between sonB1 and mutants of the *A. nidulans* NBS1 orthologue *scaA^{NBS1}* (Brushi et al. 2001). Notably, sonB1 mutants were not viable at 42° when combined with the *scaA1^{NBS1}* mutation, even without addition of any genotoxic agent (Figure 4A). Although sonB1 *scaA1^{NBS1}* double mutants formed smaller colonies at 32° and 37° than either single mutant, the synthetic lethality was restricted to 42°, the same temperature at
which sonB1 mutants display DNA damage sensitivity (Figure 4A). The scaA1 mutation is predicted to result in a truncated SCAANBS1 protein due to a single base transversion introducing a stop codon in the scaA1 reading frame (BRUSHI et al. 2001). To determine if the temperature dependent synthetic lethality between sonB1 and scaANBS1 was due to complete loss of SCAANBS1 function, we constructed a double mutant between sonB1 and a null allele of scaA (SEMIGHINI et al. 2003). The sonB1ΔscaANBS1 and sonB1scaANBS1 double mutants behaved identically confirming that SCAANBS1 function is required for sonB1 survival at 42°.

SCAANb1s is a component of the MRN complex which consists of MRE11, RAD50 and NBS1 (SEMIGHINI et al. 2003; for review see D’AMOURS and JACKSON 2002; D’ADDA Di FAGAGNA et al. 2004; STAVRIDI and HALAZONETIS 2005; ZHANG et al. 2006). We next determined if sonB1 displayed a similar synthetic lethal interaction with a disrupted allele of mreAMRE11, the A. nidulans orthologue of MRE11 (SEMIGHINI et al. 2003). In contrast to the synthetic lethality observed with scaANBS1 mutants, sonB1 mreAMRE11 double mutants were able to form a colony at 42° (Figure 4C). As MRE11 encodes the DNA nuclease, strand annealing and strand dissociation activity of the MRN complex (SEMIGHINI et al. 2003; for review see D’AMOURS and JACKSON 2002), this suggests that the synthetic lethality between sonB1 and the scaANBS1 mutants at 42° is independent of these MRN complex activities.

Both the sonA1 and sonB1 NPC mutants suppress the nimA1 G2 arrest at 42°. SONAGle2 and SONBnNup98 directly bind each other and the mutations in these genes which suppress nimA1 likely do so by similar mechanisms (DE SOUZA et al. 2003). However, these NPC mutants differ in that only sonB1, and not sonA1, is sensitive to DNA damaging agents at 42° (Figure 1). We therefore determined if the sonA1 mutation genetically interacted with MRN complex mutants in a similar manner to sonB1 at 42°. As with sonB1 mutants, sonA1 mutants did not display
synthetic lethality with the mreA<sup>MRE11</sup> disruption at 42° (Figure 4C). Additionally, and in contrast to the synthetic lethality between sonB<sup>1</sup> and scaA<sup>NBS1</sup> mutants at 42°, both the sonA<sup>1</sup> scaA<sup>NBS1</sup> null and sonA<sup>1</sup> scaA<sup>NBS1</sup> double mutants were viable at 42° (Figure 4A and B). Together, these data suggest that sonB<sup>1</sup> synthetic lethality with the scaA<sup>Nbs1</sup> mutants at 42° is likely independent of the sonB<sup>1</sup> mutant defect that suppresses a nimA<sup>1</sup> G2 arrest. Rather, sonB<sup>1</sup> scaA<sup>NBS1</sup> mutant synthetic lethality at 42° may result from combining the DNA damage response defect of sonB<sup>1</sup> with lack of SCAA<sup>NBS1</sup> function. However, this interpretation should be viewed with caution as the sonA<sup>1</sup> mutant displayed synthetic sickness with scaA<sup>NBS1</sup> and mreA<sup>MRE11</sup> mutants at 37° (Figure 4), perhaps suggesting a more general genetic interaction between the MRN complex and the SONA<sup>Glc2/SONBn</sup><sup>Nup98</sup> NPC subcomplex.

**Synthetic lethality between sonB<sup>1</sup> and scaA<sup>NBS1</sup> mutants at 42° is independent of the scaA<sup>NBS1</sup> checkpoint functions:** One of the functions of NBS1 is to regulate checkpoint pathways in response to DNA damage and this function is conserved for A. nidulans SCAA<sup>NBS1</sup> (SEMIGHINI et al. 2003). The role of NBS1 in checkpoint regulation involves the ATR/ATRIP complex and/or ATM and ultimately prevents mitotic entry by tyrosine 15 phosphorylation of Cdc2 (for review see ZHOU and ELLEDGE 2000). We rationalized that if loss of checkpoint function in scaA<sup>NBS1</sup> mutants was causing synthetic lethality with sonB<sup>1</sup> mutants, sonB<sup>1</sup> mutants should also show synthetic lethality with other checkpoint deficient mutants. However, in contrast to sonB<sup>1</sup> synthetic lethality with scaA<sup>NBS1</sup> mutants at 42°, sonB<sup>1</sup> was viable at 42° when combined with either the uvsB<sup>505</sup><sup>ATR</sup>, uvsD<sup>308</sup><sup>ATRIP</sup>, cdc2<sup>F</sup> or the ΔatmA<sup>ATM</sup> checkpoint deficient mutants (Figure 5). Therefore, it is unlikely that loss of checkpoint function in scaA<sup>NBS1</sup> mutants is causing synthetic lethality with sonB<sup>1</sup> at 42°.
Although the sonB1 mutant was viable in combination with the above checkpoint deficient mutants, sonB1 did significantly increase the DNA damage sensitivity of the uvsB505\textsuperscript{ATR}, uvsD308\textsuperscript{ATRIP}, cdc2F and the ΔatmA\textsuperscript{ATM} at 42° (Figure 5). This is consistent with the sonB1 mutation affecting a different part of the DNA damage response that the G2-DNA damage checkpoint, supporting our earlier conclusion (Figure 2).

The sonA1 nucleoporin mutant was viable at 42° in combination with all checkpoint mutants tested (Figure 5). However, interestingly, the sonA1 mutation increased the DNA damage sensitivity of the cdc2F mutant (Figure 5D). Given that the sonA1 nucleoporin mutation allows Cdc2/CyclinB into the nucleus (Wu et al. 1998), it is likely that increased nuclear access of active Cdc2F/CyclinB in the sonA1 cdc2F double mutant is leading to increased DNA damage sensitivity. Somewhat surprisingly, the sonA1 mutation did not increase the DNA damage sensitivity of uvsB505\textsuperscript{ATR}, uvsD308\textsuperscript{ATRIP}, or ΔatmA\textsuperscript{ATM} mutants which are defective in the pathway leading to tyrosine phosphorylation of Cdc2. This may reflect the relative levels of non-tyrosine phosphorylated Cdc2 in uvsB505\textsuperscript{ATR}, uvsD308\textsuperscript{ATRIP}, or ΔatmA\textsuperscript{ATM} mutants compared with that of the cdc2F mutant under these conditions.

**The sonB1 mutation does not cause DNA damage:** Another possibility to explain the synthetic lethal interaction between sonB1 and scaA\textsuperscript{NBS1} mutants is that the sonB1 mutation itself leads to DNA double strand breaks which are not repaired in the absence of SCAA\textsuperscript{NBS1} function. If this were the case, sonB1 mutants should also display synthetic lethality at 42° with mutants deficient in the repair of DNA double strand breaks. DNA double strand breaks are repaired by either homologous recombination or non-homologous end joining (NHEJ). In *A. nidulans*, the *uvuC\textsuperscript{Rad51}* gene encodes a Rad51 orthologue and *uvuC\textsuperscript{Rad51}* mutants are sensitive to DNA double strand breaks and display defects in homologous recombination (Van Heemst et al. 1997; Chaé...
and KAFCR, 1997; SEONG et al. 1997; ICHIOKA et al. 2001). We generated the sonB1 uvsC114\textsuperscript{Rad51} double mutant which was viable at 42\(^\circ\) (Figure 6A) arguing that the sonB1 mutation is not leading to DNA double strand breaks. Similarly deletion of nkuA\textsuperscript{Ku70}, the \textit{A. nidulans} orthologue of the Ku70 gene which functions in NHEJ in other systems (for review see HOPFNER et al. 2002) had no effect on sonB1 viability at 42\(^\circ\) (Figure 6B). However, surprisingly the nkuA\textsuperscript{Ku70} null displays no sensitivities to DNA damaging agents indicating that Ku70 mediated NHEJ may only play a minor role in the DNA damage response in \textit{A. nidulans} or that there is a second NHEJ pathway functioning without nkuA\textsuperscript{Ku70} (NAYAK et al. 2006). We next tested the viability of the sonB1 mutation when combined with the \textit{A. nidulans} uvsH77\textsuperscript{Rad18} postreplication repair deficient mutant (YOO\textit{N} et al. 1995). The sonB1 uvsH77\textsuperscript{Rad18} double mutant was viable at 42\(^\circ\) further supporting that the sonB1 mutation was not causing DNA damage (Figure 6C).

Interestingly, sonB1 uvsC114\textsuperscript{Rad51} and sonB1 uvsH77\textsuperscript{Rad18} double mutants were not viable at 42\(^\circ\) in the presence of concentrations of DEO at which the respective single mutants formed viable colonies (Figure 6A and C). Moreover, this effect was specific to the sonB1 mutant and was not observed with the sonA1 mutant (Figure 6). The sonB1 nkuA null double mutant did not display any significant increase in sensitivity to DEO or camptothecin (Figure 6, data not shown) in comparison to the single mutants. These data provide further evidence that SONBn\textsuperscript{Nup98} has role in the DNA damage response and indicates that this function is likely on a different pathway than that of either UVSC\textsuperscript{Rad51} or UVSH\textsuperscript{Rad18}.

\textit{γ-H2AX phosphorylation has a role in the DNA damage response independent of SONBn}\textsuperscript{Nup98}: We have previously shown that the histone H2A/H2B gene pair acts as a copy number suppressor of sonB1 cold sensitivity and sensitivity to hydroxyurea at 32\(^\circ\), but has no effect on the sonA1 mutant (De SOUZA et al. 2003). Phosphorylation of a conserved serine in the
histone H2AX variant (to generate $\gamma$-H2AX) in nucleosomes located near sites of DNA double strand breaks has important roles in the DNA damage response including regulating MRN complex localization to sites of DNA damage (KOBAYASHI et al. 2002; CELESTE et al. 2003; for review see VIDANES et al. 2005). This conserved serine of histone H2AX isoforms is present near the C-terminus of the single A. nidulans histone H2A gene (Figure 7A) (MAY and MORRIS 1987) which we term H2AX. Because of the genetic interaction between sonB1 and histone H2AX/H2B, and of the role of $\gamma$-H2AX phosphorylation for MRN complex function, we determined the effect of preventing $\gamma$-H2AX phosphorylation on sonB1 mutants at 42°C. We endogenously replaced the conserved serine in the C-terminal of H2AX with alanine to generate a H2AX-S129A mutant which was viable. The DNA damage sensitivity of the H2AX-S129A mutant was compared to mutants in the $uvSB^{ATR}$ and $atmA^{ATM}$ PIKK kinases, orthologues of which phosphorylate H2AX in other systems (DOWNS et al. 2000; CELESTE et al. 2003; NAKAMURA et al. 2004). The H2AX-S129A mutant was sensitive to DEO, and camptothecin (Figure 7B) demonstrating for the first time in A. nidulans that serine 129 is required for the DNA damage response, and is likely phosphorylated in response to DNA damage. The H2AX-S129A mutant displayed no sensitivity to hydroxyurea (Figure 7B) indicating that serine 129 is not essential for the response to a slow S-phase in A. nidulans. The H2AX-S129A mutant was less sensitive to DNA damaging agents than the $uvSB^{5^{ATR}}$ mutant, consistent with UVSB$^{ATR}$ having roles in the DNA damage response in addition to $\gamma$-H2AX phosphorylation. In contrast to $uvSB^{5^{ATR}}$, the $atmA^{ATM}$ null was only more sensitive than the H2AX-S129A mutant in the presence of camptothecin which leads to the formation of DNA double strand breaks (Figure 7B). This is consistent with ATM function being more specific for response to DNA double...
strand breaks and that ATM is required for events in addition to H2AX-S129 phosphorylation during double strand break repair (MALAVAZI et al. 2006).

We next assessed if preventing H2AX-S129A phosphorylation had any effect on the sonB1 mutant. The sonB1 H2AX-S129A double mutant was not synthetically lethal at 42° and therefore H2AX phosphorylation is not essential for viability of sonB1 mutants at 42°. Notably, sonB1 H2AX-S129A double mutants displayed a much greater sensitivity to both DEO and camptothecin at 42° compared to either single mutant (Figure 7C). This effect was specific for sonB1 as sonA1 H2AX-S129A double mutants did not display any such additive effects (Figure 7C). These data are consistent with sonB1 functioning on a different pathway of the DNA damage response than that leading to H2AX-S129 phosphorylation.
DISCUSSION

Here we report that the sonB1 nucleoporin mutant is highly sensitive to DNA damaging agents specifically at 42\(^\circ\) indicating that this mutation causes a defect in the DNA damage response at 42\(^\circ\). As the sonB1 mutation also suppresses a nimA1 G2 arrest at 42\(^\circ\) (De Souza et al. 2003), we hypothesized that sonB1 mutants may have a defective G2-DNA damage checkpoint resulting in cells entering mitosis without repair of DNA damage. Supporting this, we found that sonB1 germlings which had entered the cell cycle were sensitive to UV irradiation but sonB1 quiescent conidiospores were not. This differential in the UV sensitivity of germlings and conidiospores is similar to that seen for A. nidulans mutants which are unable to tyrosine phosphorylate Cdc2 causing a defective G2-DNA damage checkpoint (Ye et al. 1997; De Souza et al. 1999). However, we surprisingly found that in sonB1 mutants Cdc2 is tyrosine phosphorylated and cells arrest in G2 normally in response to DNA damage, indicating that the G2-DNA damage checkpoint is functional. Further, sonB1 cdc2F double mutants were more sensitive to DNA damaging agents than the respective single mutants providing genetic evidence that SONBn\(^{\text{Nup98}}\) functions on a different pathway in the DNA damage response than that leading to Cdc2 tyrosine phosphorylation. Therefore, the DNA damage sensitivity of the sonB1 mutant at 42\(^\circ\) is independent of the G2-DNA damage checkpoint.

It has become clear that the MRN complex, which consists of MRE11, RAD50 and NBS1, is a key player in the DNA damage response (for review see D’Amours and Jackson 2002; Stavridi and Halazonetis 2005; Zhang et al. 2006). The MRN complex initially functions early in the DNA damage response together with the ATM and ATR kinases to regulate signaling and checkpoint pathways and then later in the DNA damage response to help facilitate homologous recombination and NHEJ (Difilippantonio et al. 2005; Jazayeri et al. 2006).
2006; Lee and Paull, 2005; Stiff et al. 2005; Uziel et al. 2003; You et al. 2005; for review see Abraham and Tibbetts, 2005; Stavridi and Halazonetis 2005; Zhang et al. 2006). The most striking finding of this study was the temperature dependent, synthetic lethal interaction between sonB1 and either the scaA1^{NBS1} mutant or the scaA^{NBS1} null. This synthetic lethality was restricted to 42°C, the same temperature at which sonB1 displays high DNA damage sensitivity. Although sonB1 mutants are defective in some aspect of G2 regulation at 42°C, we do not believe this is the defect causing synthetic lethality with scaA^{NBS1} mutants. This is because while both the sonA1 and sonB1 nucleoporin mutants suppress a nimA1 G2 arrest at 42°C (Wu et al. 1998; De Souza et al. 2003), only sonB1 and not sonA1, displays DNA damage sensitivity and is completely dead in combination with scaA^{NBS1} mutants at 42°C. This argues that sonB1 synthetic lethality with scaA^{NBS1} mutants is likely not due to sonB1 G2 regulation defects at 42°C, but rather due to the defect of the sonB1 mutant in the DNA damage response. Our data therefore suggest that it is a combination of the sonB1 DNA damage response defect and lack of SCAA^{NBS1} function that causes synthetic lethality.

One potential link between sonB1 and the MRN complex is the histone H2AX/H2B gene pair. We previously isolated histone H2AX/H2B as a copy number suppressor of sonB1 cold sensitivity and hydroxyurea sensitivity at 32°C (De Souza et al. 2003). However, the high level of DNA damage sensitivity of sonB1 mutants is not suppressed by extra copy histone H2AX/H2B (data not shown). Phosphorylation of H2AX by the ATM/ATR kinases to generate γ-H2AX is important for the localization of the NBS1 to sites of DNA damage (Kobayashi et al. 2002; Celeste et al. 2003). This function is likely conserved in A. nidulans as we found that the H2AX-S129A mutant was sensitive to DNA damaging agents and it has been shown that SCAA^{NBS1} accumulates in the nucleus in an UVSB^{ATR} dependent manner in response to DNA
damage (FAGUNDES et al. 2005). However, we found that sonB1 H2AX-S129A double mutants were viable at 42° suggesting that it is not a defect in NBS1 localization to γ-H2AX that is causing synthetic lethality between sonB1 and the scaANBS1 mutants. Further, the marked increase in DNA damage sensitivity of sonB1 H2AX-S129A double mutants relative to the respective single mutants suggests that SONBnNup98 and γ-H2AX phosphorylation are on different pathways of the DNA damage response.

Intriguingly, the sonB1 mutant was viable at 42° when combined with a disrupted allele of mreAMRE11, the A. nidulans orthologue of mre11 which encodes the DNA nuclease, strand annealing and strand dissociation activities of the MRN complex (SEMIGHINI et al. 2003; for review see D’AMOURS and JACKSON 2002; ZHANG et al. 2006). These MRE11 DNA modifying activities are likely important for the DNA repair functions of the MRN complex. Therefore, sonB1 synthetic lethality is likely independent of the DNA repair functions of the MRN complex which is further supported by our finding that the sonB1 mutant was not synthetically lethal with other DNA repair mutants at 42°. One NBS1 function which is independent of MRE11 is NBS1 binding to the γ-H2AX phospho-serine epitope via the FHA/BRCT domain located in the N-terminal of NBS1 (KOBAYASHI et al. 2002; for review see ZHANG et al. 2006). While we have shown that γ-H2AX phosphorylation is not required for survival of sonB1 mutants at 42°, it is likely that the NBS1 FHA/BRCT domain also binds other as yet unidentified phospho-serine epitopes. Given our data, it is tempting to speculate that SCAA NBS1 binding to phospho-serine epitopes may be required for survival of sonB1 mutants at 42°. Although the FHA/BRCT domain of NBS1 orthologues is not well conserved, a recent bioinformatics study suggests that SCAA NBS1 contains the conserved residues required for binding to phospho-serine epitopes (BECKER et al. 2006).
Our data suggest that the loss of cell cycle checkpoint functions which occurs in $scaA^{NBS1}$ mutants (SEMIGHINI et al. 2003) is not the defect in these mutants causing synthetic lethality in combination with $sonB1$ mutants at $42^\circ$. This is because the $sonB1$ mutation is not synthetically lethal in combination with mutations in other cell cycle checkpoint regulators including the $A. nidulans$ orthologues of $ATR$, $ATRIP$, $ATM$ or the $cdc2F$ mutant (DE SOUZA et al. 1999; HOFMANN and HARRIS 2000; YE et al. 1997; MALAVAZI et al. 2006). In addition, given that the $sonB1$ mutant was viable when combined with these cell cycle checkpoint mutants or the $uvSC^{Rad51}$ or $uvSH^{Rad18}$ DNA repair mutants, it is unlikely that $sonB1$ is in itself causing DNA damage at $42^\circ$. Notably, however, as double mutants between $sonB1$ and $uvSC^{Rad51}$ or $uvSH^{Rad18}$ mutants were more sensitive to DEO than the respective single mutants, it is likely that $SONBn^{Nup98}$ functions on a different pathway in the DNA damage response than with $UVSC^{Rad51}$ or $UVSH^{Rad18}$.

Interestingly, the NPC has been demonstrated to have roles in tethering telomeres to the nuclear periphery in budding yeast (GALY et al. 2000; FEUERBACH et al. 2002; THERIZOLS et al. 2006). Further, a recent study has shown that anchoring of telomeres to the nuclear periphery is required for efficient DNA double strand break repair (THERIZOLS et al. 2006). These authors found that nucleoporin mutants which failed to properly tether telomeres to the nuclear periphery display a decreased efficiency in the repair of DNA double strand breaks induced proximal to telomeres. It will therefore be interesting to determine if telomeric localization and/or function is disrupted in $sonB1$ mutants and whether this contributes to the DNA damage sensitivity of $sonB1$ mutants. Further, given that the MRN complex functions in telomere regulation (VerdUN et al. 2005; for review see D'ADDA DI FAGAGNA et al. 2004), it is possible that the synthetic lethality between $sonB1$ and $scaA^{NBS1}$ mutants may be due to combining different defects in telomere
biology caused by these mutations.

Another explanation for the DNA damage sensitive phenotype of *sonB1* is that some aspect of nucleocytoplasmic transport required for the damage response is not functioning in this mutant at 42°. We consider this unlikely given that nuclear transport of a nuclear localization sequence (NLS) reporter construct is normal in *sonB1* mutants at 42° (CDS and SAO unpublished observations) and that the *sonA1* nucleoporin mutant does not display similar DNA damage sensitivities. However, given that the recruitment of the MRN complex to sites of DNA damage has been reported to require relocation of MRE11 and RAD50 from the cytoplasm to the nucleus (Tauchi *et al*. 2001; Kobayashi *et al*. 2002), we cannot rule out the involvement of SONBn{	extsuperscript{Nup98}} in regulating specific nuclear transport pathways during the DNA damage response.

Null alleles of certain budding yeast nucleoporins display sensitivity to DNA damaging agents (Bennett *et al*. 2001; Chang *et al*. 2002; Galy *et al*. 2000; Loell et al. 2005; Therizols *et al*. 2006) however, we know of no single amino acid substitutions in yeast nucleoporins which cause sensitivity to DNA damaging agents. Similar to the case for *sonB1*, the DNA damage sensitivity of budding yeast nucleoporin nulls does not appear to be caused by general defects in DNA repair (Loell et al. 2005; Therizols *et al*. 2006). Interestingly genome wide screens have revealed that null alleles of the budding yeast *nup120* and *nup133* nucleoporins, which are sensitive to DNA damaging agents, display synthetic lethality with null alleles of MRN (MRX in budding yeast) coding genes (Loell et al. 2005). While the mechanism of this interaction has not been established, it suggests that the genetic interaction between the NPC and the MRN complex is likely conserved.

Notably, of the thirteen nucleoporins which are non-essential in *A. nidulans*, none display obvious sensitivities to DNA damaging agents (unpublished observations). This makes it even
more intriguing that a single amino acid substitution in the essential $sonB^{Nup98}$ nucleoporin gene causes conditional DNA damage sensitivity at 42°. Most DNA damage sensitive mutations identified to date in simple organisms occur in non-essential genes. This is because the genetic screens used to identify them require mutations to be viable but display sensitivity to DNA damaging agents. This fact has selected for DNA damage sensitive mutations in non-essential genes. Screens for conditional DNA damage sensitive mutations, similar to the heat dependent DNA damage sensitivity of the $sonB1$ mutation, may therefore identify essential genes which function in novel aspects of the DNA damage response.

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FIGURE 1. The sonB1 mutant is highly sensitive to DNA damaging agents at 42°. (A) Colony formation of wild type (R153), uvsB505ATR (CDS314), cdc2F (FRY24), sonA1 (CDS365) and sonB1 (CDS364) strains under the indicated conditions. Plates were incubated for 3 days. Note, sectoring of the cdc2F mutant at 32° in the presence of DEO or camptothecin is due to loss of the cdc2F allele by plasmid eviction leaving the wild type allele. (B) Viability of the indicated strains (250 conidiospores spread per plate, 2 plates per strain) at 32° and 42° in the presence of different concentrations of the DNA alkylating agent DEO. Viability was assessed after 3 days incubation.

FIGURE 2. Differential UV sensitivity of sonB1 quiescent conidiospores and germlings at 42°. Conidiospores (250 per plate, two plates per strain) of wild type (GR5), cdc2AF (FRY20-1), sonB1 (CDS40) and uvsH304Rad18 (A329) were spread on plates and either allowed to germinate at (A) 32° or (B) 42° for 6 hr prior to UV irradiation, or (C) immediately UV irradiated. After irradiation, plates were incubated at the indicated temperatures for two days to allow colony formation. The percent survival after UV irradiation is expressed as the percentage of colonies produced in the absence of treatment. This experiment was performed twice with similar results.

FIGURE 3. sonB1 mutants undergo tyrosine phosphorylation of Cdc2 and arrest in G2 in response to DNA damage. (A and B) Log phase wild type (GR5), sonB1 (CDS40), nimA5 (SO6) and nimA5 sonB1 (CDS119) cultures were shifted to 42° for 3 hr. Cultures were then divided into two and grown for another 2 hr in the presence or absence of 0.04% MMS. The relative levels of tyrosine 15 phosphorylated Cdc2 was determined by immunoblotting with an antibody specific for this epitope at the indicated time points. Levels of tubulin are shown as a loading
control. (C) Chromosome mitotic index (CMI) of nimA5 (SO54), nimA5 cdc2AF (AT27) and nimA5 sonB1 (CDS119) germlings treated or not with 0.025% MMS during a nimA5 G2 arrest (42°C) prior to release to nimA5 permissive temperature (32°C) in the absence of MMS. Note, nimA5 and nimA5 + sonB1 cells did not display an increase in CMI % even after 30 min following release from nimA5 arrest in the presence of MMS. (D) Wild type (GR5) and sonB1 (CDS40) conidiospores were germinated in the presence or absence of 0.01% MMS and the CMI determined by DAPI staining. Nocodazole (5 μg/ml) was included to prevent mitotic exit once cells entered mitosis.

**FIGURE 4.** The sonB1 mutant is synthetically lethal with scaA^{NBS1} mutants at 42°C. Wild type and the indicated single or double mutants were inoculated on plates and grown at either 37°C (2 days) or 42°C (3 days). Note that sonB1 displays synthetic lethality with either (A) scaA1^{NBS1} or (B) the scaA^{NBS1} null at 42°C but not with the (C) mreAMRE11 mutant. Strains used were CDS323, CDS324, CDS326, CDS350, CDS351, CDS364, CDS365, CDS373, CDS374, CDS375, MKF11 and R153 and are listed in Supplemental Table S1.

**FIGURE 5.** The sonB1 mutant is not synthetically lethal with checkpoint deficient mutants at 42°C. Wild type and the indicated single or double mutants were inoculated on plates and grown at either 37°C (2 days) or 42°C (3 days). Colony formation of strains in the presence of the DNA damaging agents DEO or camptothecin was also evaluated as indicated. Strains used were A574, CDS204, CDS207, CDS293, CDS314, CDS319, CDS320, CDS353, CDS364, CDS365, CDS366, CDS367, CDS369, FRY24 and R153 and are listed in Supplemental Table S1.
Figure 6. The sonB1 mutant is not synthetically lethal with DNA repair deficient mutants at 42°C. (A-C) Wild type and the indicated single or double mutants were inoculated on plates and grown at either 37°C (2 days) or 42°C (3 days). Colony formation of strains in the presence of the DNA damaging agent DEO was also evaluated as indicated. Strains used were CDS260, CDS261, CDS311, CDS315, CDS330, CDS352, CDS368, CDS370, CDS364, CDS365, R153 and TN02 are listed in Supplemental Table S1.

Figure 7. γ-H2AX phosphorylation is required for the DNA damage response in A. nidulans but not for viability of sonB1 mutant at 42°C. (A) Alignment (ClustalW, Biology Workbench: http://workbench.sdcs.edu/) of the C-terminal tail of H2AX from the indicated organisms. The serine in the conserved SQE motif is indicated in a box. Identical (*) and conserved groups (:) are indicated. (B) DNA damage sensitivities of wild type (R153), H2AX S129A (CDS198), uvsB505ATR (CDS314) and atmATM null (CDS320) strains at 32°C. (C) Colony formation of wild type and the indicated single or double mutants at 32°C and 42°C in the presence or absence of 0.005% DEO or 2 μg/ml camptothecin. Strains used were CDS198, CDS200, CDS201, CDS364, CDS365, and R153 and are listed in Supplemental Table S1.
Figure 1 De Souza et al.
Figure 2  De Souza et al.
Figure 3  De Souza et al.
Figure 4  De Souza et al.
Figure 5 De Souza et al.
Figure 6  De Souza et al.
A. Human H2AX

S. cerevisiae H2A.1
S. cerevisiae H2A.2
S. pombe H2A.1
S. pombe H2A.2
A. nidulans H2AX

** Wild Type H2A**

** sonB1**

** sonA1**

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B. Wild Type S129A uvsB505 ΔatmA

Control

0.02% DEO

25 ug/ml Camp

8 mM HU

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C. Wild Type H2A

sonB1 + S129A

sonA1 + S129A

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Figure 7 De Souza et al.