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Initiation of DNA damage responses through XPG-related nucleases

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Lesion-specific enzymes repair different forms of DNA damage, yet all lesions elicit the same checkpoint response. The common intermediate required to mount a checkpoint response is thought to be single-stranded DNA (ssDNA), coated by replication protein A (RPA) and containing a primer-template junction. To identify factors important for initiating the checkpoint response, we screened for genes that, when overexpressed, could amplify a checkpoint signal to a weak allele of *chk1* in fission yeast. We identified Ast1, a novel member of the XPG-related family of endo/exonucleases. Ast1 promotes checkpoint activation caused by the absence of the other XPG-related nucleases, Exo1 and Rad2, the homologue of Fen1. Each nuclease is recruited to DSBs, and promotes the formation of ssDNA for checkpoint activation and recombinational repair. For Rad2 and Exo1, this is independent of their S-phase role in Okazaki fragment processing. This XPG-related pathway is distinct from MRN-dependent responses, and each enzyme is critical for damage resistance in MRN mutants. Thus, multiple nucleases collaborate to initiate DNA damage responses, highlighting the importance of these responses to cellular fitness.

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Introduction

DNA damage comes in many chemical forms, ranging from perturbations in one strand of an intact DNA molecule to double-stranded DNA breaks (DSBs). Given the various different types of DNA damage, there are many independent modes used by the cell to detect and repair lesions in order to ensure continuity of the genome. The cellular response to DNA damage frequently needs to activate a cell-cycle checkpoint, a delay that allows time for DNA repair to be completed prior to commitment to S phase or mitosis. Unlike the specialized repair pathways, there are a limited number of DNA damage checkpoints. These include p53-dependent responses, and the more ancient p53-independent responses that delay progression through G2 phase via inhibition of the mitotic cyclin-dependent kinase (O'Connell *et al*, 2000; Kuntz and O'Connell, 2009).

In order to dissect the mechanisms of checkpoint regulation, several groups have identified genes in screens for loss-of-function mutants through their inability to delay mitosis following DNA damage (Hartwell and Weinert, 1989; Al-Khodairy and Carr, 1992; Al-Khodairy *et al*, 1994; Weinert *et al*, 1994). These screens have identified a large number of highly conserved genes, leading to a detailed model of G2 DNA damage checkpoint control that is effectively identical from the fission *Schizosaccharomyces pombe* (the model used in this study) through to humans. Following DNA damage, inhibition of the mitotic cyclin-dependent kinase prevents entry into mitosis. This is mediated by Chk1, the effector kinase of the DNA damage checkpoint. In order for this pathway to signal a delay to the onset of mitosis, all lesions must first be processed into a common intermediate, which is believed to be single-stranded DNA (ssDNA) with a 3′-overhang that is coated by replication protein A (RPA) and contains a primer-template junction (O'Connell and Cimprich, 2005; MacDougall *et al*, 2007). The RPA-coated ssDNA then acts as a scaffold for the assembly of checkpoint complexes comprising the PCNA-related 9-1-1 complex and the PI3K-related kinase ATR (Rad3 in *S. pombe*) along with its targeting subunit ATRIP (Rad26 in *S. pombe*). Subsequently, mediator proteins are recruited, which in turn recruit Chk1 for activating phosphorylation by ATR.

While a detailed understanding of the events leading to Chk1 activation have been described, what remains unclear are the events that occur in between lesion detection and the processing of a lesion into the template on which these complexes are assembled. This checkpoint template also acts as the initial structure used for the repair of the lesion through homologous recombination (HR) (Krogh and Symington, 2004). Therefore, while mutants that lead to a defect in cell-cycle delay define the downstream components of the checkpoint-signalling pathway, a defect in the initiating events of this pathway will also block DNA repair. However, it is unknown whether the amount of ssDNA produced is equivalent for checkpoint signalling and repair by HR (Symington and Gautier, 2011).

To date it would appear that simple loss-of-function screens for checkpoint mutants defined by single genes have reached saturation, at least in the yeasts *S. pombe* and *Saccharomyces cerevisiae*. Therefore, the genes involved in checkpoint initiation are unlikely to be defined by such approaches. An emerging model of lesion resection involving a multi-step processing of DSBs initiated by the Mre11-Rad50-Nbs1 (MRN) complex and CtIP/Ctp1/Sae2 has been described. Additional resection is carried out through multiple different pathways including those utilizing RecQ helicases, and the nucleases, Exo1 and Dna2 (Nakada *et al*, 2004; Limbo *et al*, 2007; Sartori *et al*, 2007; Gravel *et al*, 2008; Liao *et al*, 2008; Mimitou and Symington, 2008; Zhu *et al*, 2008; Mimitou and

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Symington, 2009). Defects in these genes impacts on recombination, however, Chk1 activation still occurs in the absence of MRN (Grenon *et al*, 2001; Willis and Rhind, 2010; Limbo *et al*, 2011), Ctp1 (Limbo *et al*, 2007; Sartori *et al*, 2007) and Exo1 (Gravel *et al*, 2008 and see below) suggesting other mechanisms of checkpoint activation must exist.

In an effort to uncover new factors that function in the DNA damage response, we reasoned that the overexpression of checkpoint activating enzymes would enable the amplification of a checkpoint response. To this end, we searched for high copy suppressors of a weak allele of *chk1* in *S. pombe* (Latif *et al*, 2004; Kosoy and O'Connell, 2008). This identified *ast1*, a homologue of *Drosophila Asteroid* and Human *ASTE1*, which encode uncharacterized members of the XPG-related family of endo/exonucleases. This family also includes the major Okazaki fragment processing enzyme Flap Endonuclease 1 (Fen1 in humans, Rad2 in *S. pombe* and Rad27 in *S. cerevisiae*) and Exonuclease 1 (Exo1). Previous studies have implicated these enzymes in DNA repair pathways, particularly during DNA replication, but they are not required for the activation of Chk1. We show here that this family of nucleases collaborates to generate ssDNA. Ast1 appears to be crucial in mounting a checkpoint response to endogenous damage in cells lacking Rad2 and Exo1. In turn, Rad2 and Exo1 are required to resect lesions during G2 phase. Each of these XP-G-related nucleases is required for damage resistance in cells lacking the MRN complex. The redundancy between these enzymes in checkpoint initiation, and the multiple, unique checkpoint-independent functions of each, explains why they have not been implicated previously in checkpoint activation.

Results

Ast1 overexpression amplifies a Chk1-activating signal

We have previously described several temperature-sensitive (ts) alleles of *chk1*. One of these, *chk1-E472D*, is functional at 25°C but is not activated by DNA damage induced phosphorylation of S345 by Rad3 (the ATR homologue) at 36°C (Latif *et al*, 2004; Kosoy and O'Connell, 2008). Biochemical characterization of Chk1 activation kinetics has shown that the duration of Chk1 activity, but not its specific activity, is dose dependent, suggesting that it exists in either an 'on' or 'off' state (Latif *et al*, 2004; Tapia-Alveal *et al*, 2009). In order to facilitate a screen for new checkpoint genes, we aimed to find a condition of Chk1 dysfunction below a threshold of activity that would be amenable to being raised above this threshold by genes that could amplify an endogenous checkpoint-activating signal emanating from chromosomal lesions.

To this end, we crossed *chk1-E472D* to a number of (ts) alleles of genes involved in DNA replication, and found a tight synthetic lethality at 32.5°C of *chk1-E472D* with *cdc27-P11* (Hughes *et al*, 1992), a mutant in a subunit of DNA polymerase δ that is lethal at 36°C (Figure 1A). We exploited this synthetic lethality for a high-copy suppressor screen using a genomic library in which genes are overexpressed ~5- to 10-fold from their own promoters (Barbet *et al*, 1992). Suppressors of the double mutant *chk1-E472D cdc27-P11* were selected at 32.5°C, and suppressors of *cdc27-P11* that grew at 36°C were discarded. Plasmids that promoted growth only at 32.5°C were retained, isolated and retested. From this screen, we identified multiple isolates of *chk1, cdc1* (another subunit of Pol δ; MacNeill *et al*, 1996), and an uncharacterized gene, SPCC962.05. This gene encodes a homologue of *Drosophila Asteroid*, so named because of its proximity to the EGF signalling component *Star* (Higson *et al*, 1993). This gene is conserved in humans (*ASTE1*), but there is no apparent homologue in *S. cerevisiae*. We have therefore named this *S. pombe* gene *ast1*. These Asteroid homologues encode a member of the XPG-related nucleases, a family of endo/exonucleases, which in *S. pombe* includes Rad2 and Exo1 (Figure 1B). These nucleases differ from canonical XPG (Rad13 in *S. pombe*) in that their N-terminal (XPG-N) and Internal (XPG-I) nuclease domains lack a large spacer region that is important for substrate specificity (Dunand-Sauthier *et al*, 2005; Hohl *et al*, 2007).

Overexpression of wild-type Ast1, but not a nuclease-dead mutant, also suppressed the MMS sensitivity of *chk1-E472D* at 36°C (Figure 1C), but could not suppresses the MMS sensitivity of a kinase-dead allele of *chk1, chk1-D155A* (Figure 1D). Therefore, Ast1 is a direct suppressor of *chk1-E472D*, rather than a bypass suppressor that imposes a cell-cycle delay by an independent mechanism. Consistent with the functional data, Chk1-E472D is poorly phosphorylated by Rad3 following DNA damage (Latif *et al*, 2004), though this is partially restored by Ast1 overexpression (Figure 1E).

As Ast1 is closely related to Rad2 and Exo1 (Figure 1B), it was curious that neither of these genes were isolated in our screen. We therefore tested the suppressive activity of genomic clones of Rad2 and Exo1. Rad2 weakly suppressed the MMS sensitivity of *chk1-E472D* at 36°C (Supplementary Figure 1A), and was also a weak suppressor of the synthetic lethality of *chk1-E472D cdc27-P11*, but only at the slightly reduced temperature of 32.0°C (Supplementary Figure 1B). Conversely, genomic Exo1 showed no suppression under either condition. Thus at least for restoring activation of Chk1-E472D, Ast1 appears to have the strongest activity of the three nucleases in amplifying checkpoint signalling when overexpressed.

We then deleted *ast1*, and somewhat surprisingly, *ast1*Δ cells were not sensitive to MMS (Figure 1F) or UV-C irradiation (Figure 1G). However, at least consistent with a potential function in a Chk1-dependent pathway, *ast1*Δ did not modify the damage sensitivity of *chk1*Δ cells (Figure 1F and G).

XPG-related nucleases form a redundant family

The XPG-related enzymes possess 5′-flap endo- and 5′→3′ exonuclease activity (Lee and Wilson, 1999). Either of these activities, alone or with the help of a DNA helicase, would be capable of generating ssDNA with a 3′-overhang, the template required to assemble checkpoint complexes. However, neither *ast1*Δ (Figure 1F and G) nor *exo1*Δ (Figure 2B and C) (Tomita *et al*, 2003; Lee *et al*, 2007) cells are significantly sensitive to DNA damage. Further, while *rad2*Δ cells show several repair defects, they do not display a checkpoint defect (Murray *et al*, 1994; Klungland and Lindahl, 1997; Wu and Wang, 1999; Alseth *et al*, 2005). Therefore, we used tetrad analysis to make double and triple mutant combinations in order to assay for repair and checkpoint defects. For this analysis, we used the vital dye phloxin B to assay for cell viability 2 days after dissection, enabling phenotypes to be discerned prior to disintegration of dead cells (Figure 2A).

Double mutants between *exo1*Δ and *rad2*Δ formed micro-colonies of cell cycle-arrested (elongated) cells. These double

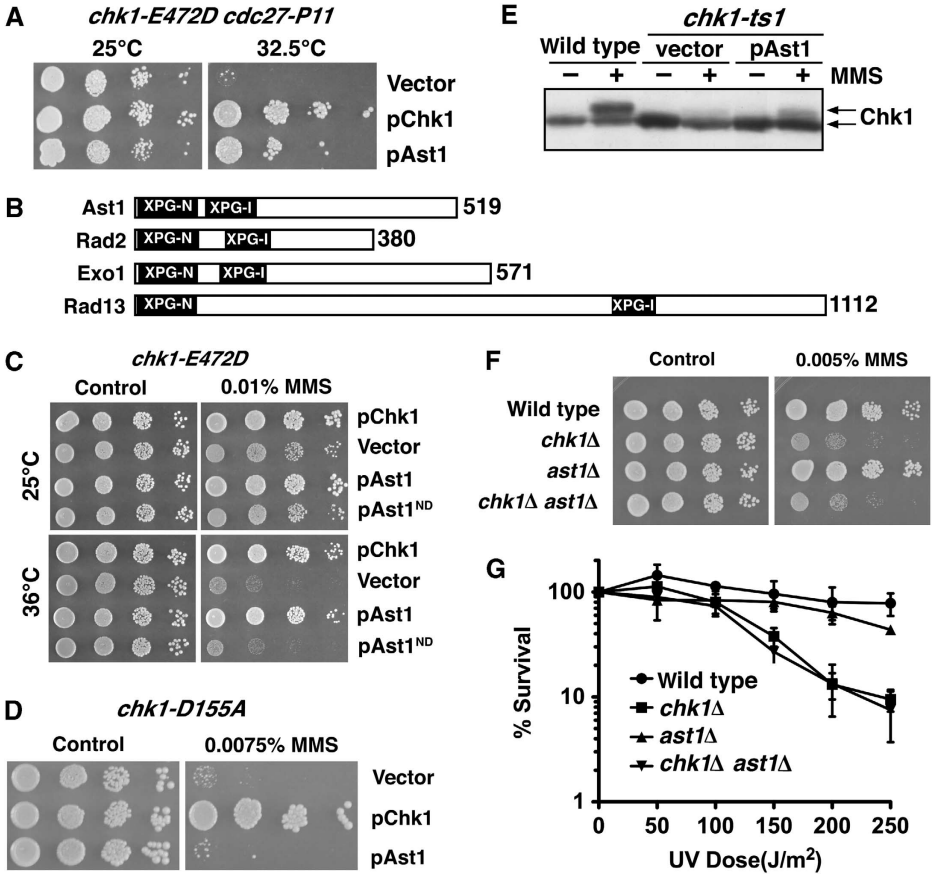


Figure 1 *ast1*, an XPG-related nuclease, is a high copy suppressor of *chk1-E472D*. (**A**) Ten-fold serial dilutions of *chk1-E472D cdc27-P11* transformed with an empty vector or the indicated plasmids and were grown at 25 and 32.5°C. A plasmid overexpressing *ast1* is able to rescue the temperature sensitivity of *chk1-E472D cdc27-P11* at 32.5°C. (**B**) A schematic of the domain structures of the XPG-related family members in *S. pombe*. The XPG-related nucleases all contain conserved N-terminal (XPG-N) and Internal (XPG-I) domains required for their nuclease activity. (**C**) Ten-fold serial dilutions of *chk1-E472D* transformed with an empty vector or the indicated plasmids (Ast1ND is the nuclease-dead derivative) were grown at 25 and 36°C in the presence and absence of 0.01% MMS. A plasmid overexpressing wild-type *ast1* is able to rescue the DNA damage sensitivity of *chk1-E472D* at 36°C. (**D**) Ten-fold serial dilutions of *chk1-D155A* transformed with an empty vector or the indicated plasmids were grown at 30°C in the presence and absence of 0.0075% MMS. A plasmid overexpressing *ast1* is unable to rescue the DNA damage sensitivity of *chk1-D155A*. (**E**) Western blot with anti-HA antibody and extracts from a wild-type strain containing HA-tagged *chk1* and from an HA-tagged-*chk1-E472D* strain transformed with an empty vector or a plasmid overexpressing *ast1*. Strains were grown in the presence and absence of 0.01% MMS. Overexpression of *ast1* restored a mobility shift to *chk1-E472D* following DNA damage. (**F**) Ten-fold serial dilutions of the indicated strains were grown in the presence or absence of 0.005% MMS at 30°C. The *chk1*Δ *ast1*Δ double mutant is not more sensitive to MMS than the *chk1*Δ single mutant. (**G**) A UV-C survival curve was performed with the indicated strains. Graph shows the mean ± s.d. for each data point ($n = 3$). The *chk1*Δ *ast1*Δ double mutant is not more sensitive to UV than the *chk1*Δ single mutant.

mutant cells were viable (phloxin negative) at this time point, though fail to go onto form colonies and are hence synthetically lethal. A similar synthetic lethality has been shown for the analogous double *exo1*Δ *rad27*Δ mutant in *S. cerevisiae*, and has been attributed to a defect in the processing of Okazaki fragments (Tishkoff *et al*, 1997). Combining *chk1*Δ with *exo1*Δ *rad2*Δ led to a terminal phenotype of dead (phloxin positive), septated cells, which is characteristic of lethal mitoses resulting from checkpoint failure (the 'cut' phenotype, where the division septum bisects the nucleus). This indicated that there is the activation of a Chk1-dependent cell-cycle arrest in the *exo1*Δ *rad2*Δ double mutant. Importantly, *ast1*Δ *exo1*Δ *rad2*Δ triple mutants died as micro-colonies containing many dead (phloxin positive) cells. These included septated (cut) cells where both daughters were dead, and those where one daughter was viable and the other dead, a phenotype characteristic of unequal chromosome segregation due to mitotic failure. Therefore, the cell-cycle arrest of *exo1*Δ *rad2*Δ cells, presumably due to replication defects, is both Chk1 and Ast1 dependent.

These data indicated that any of these three XPG-related nucleases are capable of signalling a checkpoint arrest in response to endogenous DNA damage. Consistent with this notion, the *ast1*Δ *exo1*Δ and *ast1*Δ *rad2*Δ double mutants were not more MMS (Figure 2B) or UV-C (Figure 2C) sensitive than *exo1*Δ or *rad2*Δ single mutants. Further, like many DNA repair mutants, *exo1*Δ and, in particular, *rad2*Δ cells show Chk1 activation without exogenous DNA damage, and this is significantly diminished by *ast1*Δ (Figure 2D), suggesting that endogenous levels of Ast1 are contributing to Chk1 activation in these strains.

XPG-related nucleases contribute to G2 checkpoint activation

In order to assess whether each of these three nucleases is capable of mounting a G2 checkpoint response, we needed to

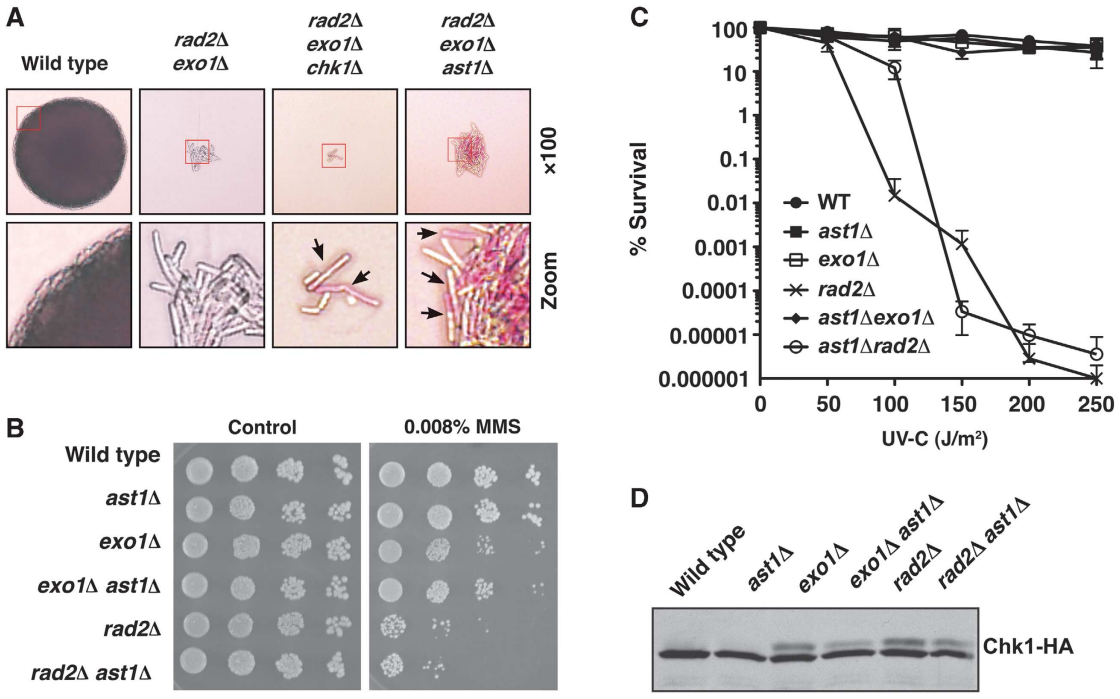


Figure 2 Lethal cell-cycle arrest of *exo1*Δ *rad2*Δ is dependent on *chk1* and *ast1*. (**A**) Single colonies from tetrad analysis are shown at the indicated magnifications, and are photographed 2 days following dissection on YES plates containing the vital dye phloxin B, which stains dead cells red. Strains are indicated above each photo. A wild-type colony is shown for comparison. The *rad2*Δ *exo1*Δ double mutant shows a cell-cycle arrest (elongated cells) that are viable (phloxin negative) at this time point, though inviable after further incubation. The *rad2*Δ *exo1*Δ *chk1*Δ triple mutant dies (phloxin positive, arrowed) with a 'cut' phenotype following 1–3 cell cycles indicating a checkpoint defect. The *rad2*Δ *exo1*Δ *ast1*Δ triple mutant dies as a micro-colony with many inviable (phloxin positive, examples arrowed) cells showing a 'cut' phenotype, also indicating a checkpoint defect. For this analysis, the 'cut' phenotype is defined as septated, phloxin-positive (dead) cells, or septated cells in which one daughter is dead (phloxin positive) and one alive (phloxin negative), which is derived from unequal chromosome segregation. (**B**) Ten-fold serial dilutions of the indicated strains were grown at 30°C in the presence and absence of 0.008% MMS. The *exo1*Δ *ast1*Δ and *rad2*Δ *ast1*Δ double mutants are not more sensitive to MMS than the *exo1*Δ and *rad2*Δ single mutants, respectively. (**C**) A UV-C survival curve was performed with the indicated strains. Graph shows the mean ± s.d. for each data point ($n = 3$). The *exo1*Δ *ast1*Δ and *rad2*Δ *ast1*Δ double mutants are not more sensitive to UV than the *exo1*Δ and *rad2*Δ single mutants, respectively. (**D**) Western blot with anti-HA antibody and extracts from strains of the indicated genotype containing HA-tagged *chk1*. The *exo1*Δ *ast1*Δ and *rad2*Δ *ast1*Δ double mutants do not have increased Chk1 activation above the levels seen in the *exo1*Δ and *rad2*Δ single mutants, respectively.

construct a conditional triple mutant. To this end, we integrated the weakest *nmt1* promoter (Basi *et al*, 1993) at the *exo1* locus to create a thiamine-repressible 'shut-off' allele (Harvey *et al*, 2004), *exo1-so*. Consistent with the tetrad analysis (Figure 2), *exo1-so* was lethal on both *rad2*Δ and *rad2*Δ *ast1*Δ backgrounds when grown on plates containing thiamine (Figure 3A). As Exo1 transcription and translation is now controlled by the *nmt1* promoter, the addition of thiamine to exponentially growing cultures caused a rapid drop in *exo1* mRNA after 2 h (Figure 3B), and an even more significant loss of Exo1 protein by 6 h (Figure 3C). Therefore, for the purposes of the experiments below, we considered *ast1*Δ *rad2*Δ *exo1-so* cells to be functionally depleted for all three XPG-related nucleases after 6 h of growth in the presence of thiamine.

Exo1 and Rad2 have been implicated in several repair mechanisms that are associated with DNA replication. These functions include, mismatch repair (MMR) (Marti *et al*, 2002), and base excision repair (BER) (Alseth *et al*, 2005) for Exo1, and UV-dependent excision (UVDE) (Yonemasu *et al*, 1997), and Smc5/6-dependent HR for Rad2 (Lehmann *et al*, 1995). These functions are in addition to the processing of Okazaki fragments by both of these enzymes. This necessitated us to develop an approach to separate the potential functions of these nucleases in mounting a checkpoint response in G2 phase from their various repair functions during DNA replication (Figure 4A). To this end, both *rad2*Δ *exo1-so* and *ast1*Δ *rad2*Δ *exo1-so* strains were crossed into a *cdc25-22* background. Cdc25 is the Cdk-activating phosphatase that is necessary for mitotic entry, and *cdc25-22* encodes an active enzyme at 25°C, but an inactive phosphatase at 36°C. *cdc25-22* cells arrest in G2 phase at 36°C, but rapidly and synchronously enter mitosis when returned to 25°C. Thus, cultures were grown at 25°C in the absence of thiamine to mid-logarithmic phase, whereupon thiamine was added for 2 h to begin Exo1 depletion. Thereafter, cultures were shifted to 36°C for a further 4 h to inactivate *cdc25-22* and fully deplete Exo1. We limited the time at 36°C to 4 h because arresting *cdc25-22* cells at 36°C for longer periods induces synthetic checkpoint defects to wild-type cells (Harvey *et al*, 2004). Finally, cells were then mock or UV-C irradiated, returned to 25°C, and the kinetics of mitotic entry measured by microscopy.

Under this protocol, irradiated wild-type cells showed a typical checkpoint-induced delay to mitotic entry that was Chk1 dependent (Figure 4B). Unirradiated *rad2*Δ *exo1-so* cells showed a highly reproducible delay to mitotic entry that was further delayed by UV-C irradiation. Time-course experiments

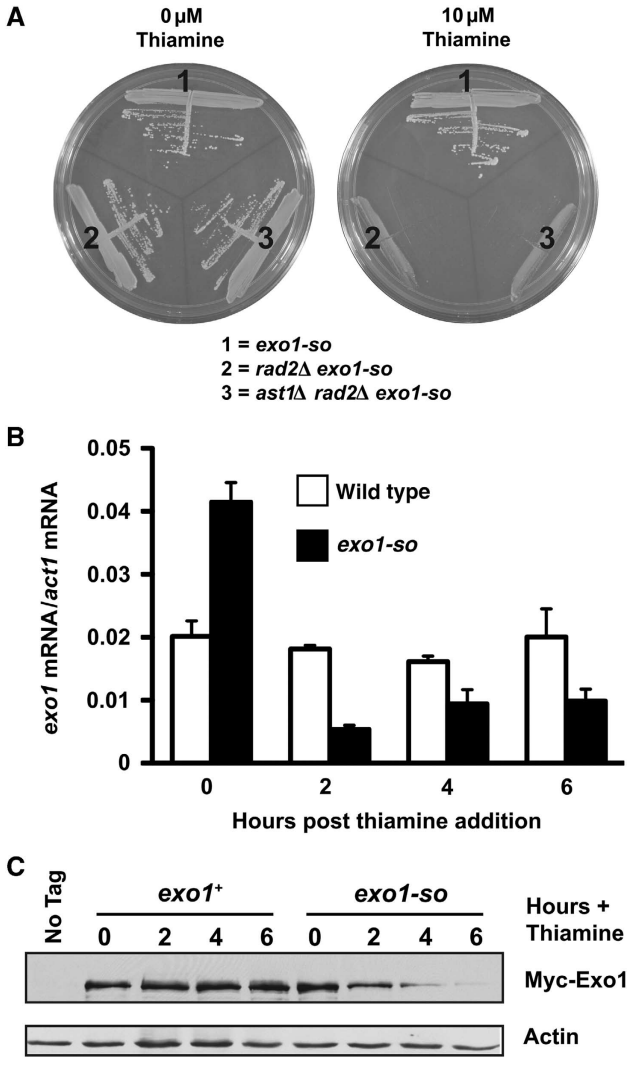


Figure 3 Creation of a thiamine repressible *exo1* shut-off allele. (**A**) The indicated strains were streaked on plates in the presence or absence of 10 μM thiamine. The *exo1-so* strain is shown as a control. In agreement with the tetrad analysis, both the double mutant *rad2*Δ *exo1-so* and the triple mutant *ast1*Δ *rad2*Δ *exo1-so* are dead in the presence of thiamine when *exo1* expression is abolished. (**B**) cDNA synthesis followed by qPCR with primers in the *exo1* open reading frame is shown for a wild-type strain and a strain containing the *exo1-so* allele. All qPCR data are normalized to data obtained with a primer in the *actin* open reading frame. Graph shows the mean ± standard error ($n = 3$–5) for each data point. By 2 h following the addition of thiamine, *exo1* expression is reduced to about 25% of wild-type levels. (**C**) Western blots using an anti-Myc antibody and an anti-Actin control with extracts from a wild-type control and cells containing a Myc-tagged *exo1-so* allele. By 6 h following the addition of thiamine, *exo1* expression is almost undetectable at the protein level.

in the single mutants confirmed this was a synthetic phenotype (Supplementary Figure 2A). While the source of the delay in unirradiated cells is not known, we think it is likely to arise from residual replication-associated defects, though gross defects were not detectable by flow cytometry (Supplementary Figure 2B and C). Conversely, *ast1*Δ *rad2*Δ *exo1-so* cells did not show the delay in unirradiated cells seen in the double mutant, which is consistent with the tetrad analysis showing Ast1 is required for the cell-cycle arrest of *rad2*Δ *exo1*Δ cells promoted by intrinsic DNA damage (Figure 2A). However, irradiated triple mutant cells also showed a significantly reduced delay compared to double mutant cells. Consistent with this, the triple mutant showed a significant enhancement of mitotic defects without irradiation as compared to the double mutant (Figure 4C). These data corroborate the tetrad analysis phenotypes arising from intrinsic DNA damage (Figure 2A), and show that the G2 checkpoint arrest in irradiated *rad2*Δ *exo1-so* cells is also reduced in the absence of *ast1*.

We prepared cell extracts from wild-type, double and triple mutants irradiated under these growth conditions, and measured the activation of Chk1 via the phosphorylation-induced mobility shift on SDS–PAGE (Walworth and Bernards, 1996). By 30 min post irradiation, wild-type cells showed Chk1 activation, but this was absent in *rad2*Δ *exo1-so* cells and just barely visible in the *ast1*Δ *rad2*Δ *exo1-so* cells (Figure 5A). Consistent with this, Chk1 kinase activity was not induced in *rad2*Δ *exo1-so* cells, and was significantly reduced in *ast1*Δ *rad2*Δ *exo1-so* cells as compared to wild type (Figure 5B).

Because Chk1 activity was lower in the double mutant compared to the triple, and yet *chk1* was required for mitotic fidelity in this strain (Figure 4C), we assayed whether the basal activity of Chk1 was important for mitotic fidelity in this strain using a non-phosphorylatable *chk1-S345A* mutant (Capasso *et al*, 2002). Indeed, mitotic defects on this background were ~25% lower than on a *chk1*Δ background (Figure 4C). Thus, there appear to be checkpoint activation mechanisms that are independent from these nucleases, and these observations also suggest that the basal activity of Chk1 is physiologically significant.

The MRN complex, with CtIP, initiates the most characterized end-resecting mechanism, where further processing occurs through a number of different enzymes. Interestingly, *rad2*Δ is synthetically lethal with *rad32*Δ in *S. pombe* (Tavassoli *et al*, 1995), which encodes the Mre11 homologue of the MRN complex. Also, while *exo1*Δ cells are not significantly sensitive to DNA damage, double mutants with *rad32*Δ are non-epistatic and are extremely sensitive to DNA damage (Tomita *et al*, 2003; Figure 5C). Further, the MMS sensitivity of *rad32*Δ cells is suppressed by *pku70*Δ, and this is dependent on *exo1*. Pku70 is the *S. pombe* homologue of Ku70, which with its partner Ku80, binds to and protects the ends of DSBs. These and similar observations in *S. cerevisiae* (Mimitou and Symington, 2010) have suggested that in the absence of MRN, Ku blocks access to DNA end resection by Exo1. However, although *pku70*Δ fails to suppress the enhanced MMS sensitivity of *rad32*Δ *exo1*Δ cells, we found that it does suppress a severe slow growth phenotype in the absence of MMS (Figure 5C). Presumably, the slow growth phenotype of *rad32*Δ *exo1*Δ cells stems from intrinsic DNA damage, and the suppression by *pku70*Δ suggests that free ends are processed by another enzyme.

Given these phenotypes, we investigated the relationship between Ast1, MRN and Pku70. As with *exo1*Δ, despite a lack of its own MMS sensitivity *ast1*Δ also greatly enhanced the MMS sensitivity of *rad32*Δ. However, this was fully suppressed by *pku70*Δ (Figure 5C), presumably via Exo1 and/or other nucleases including Rad2, though the lethality of these combinations prevented us from analysing the relevant triple mutants. Nevertheless, these observations show that

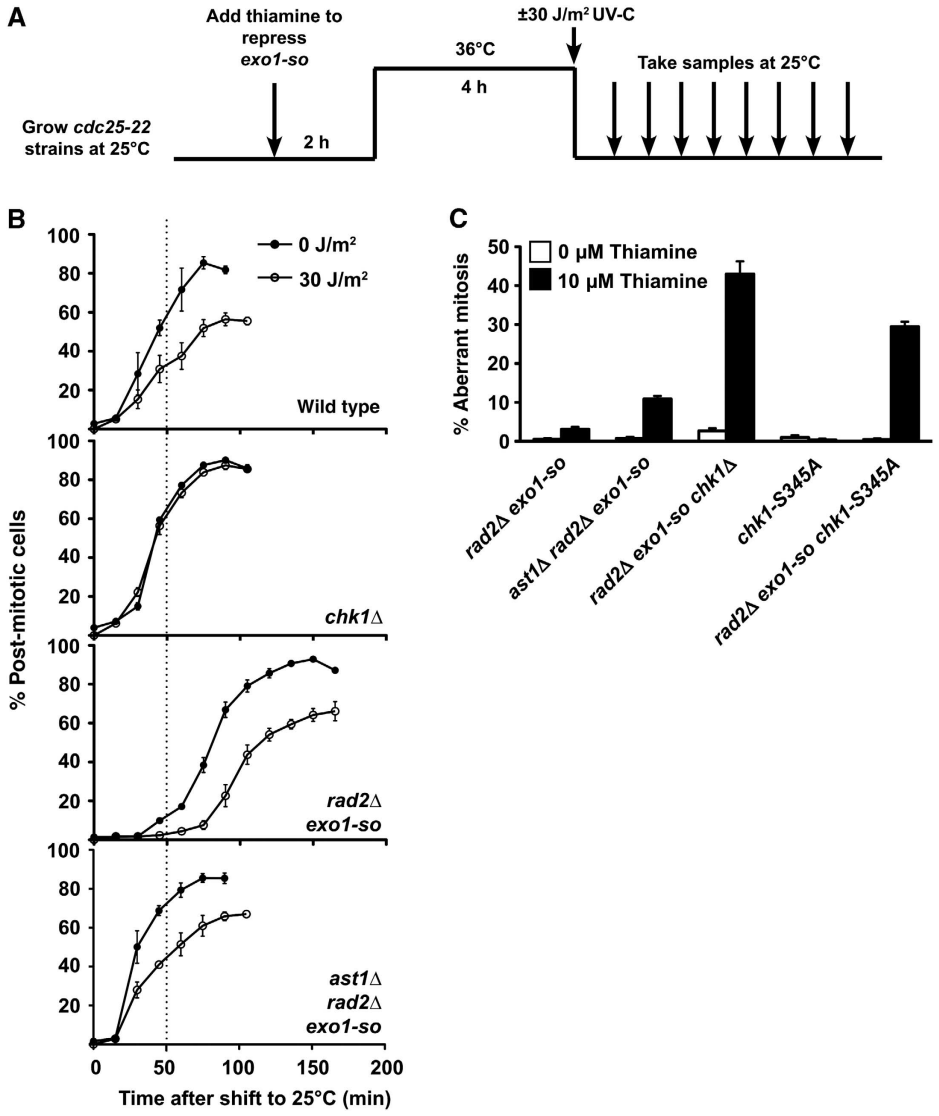


Figure 4 Checkpoint defects in strains lacking *ast1*, *exo1* and *rad2*. (**A**) Strains containing the *cdc25-22* mutation were grown at 25°C, thiamine was added to inactivate *exo1* and cells were incubated for 2 h. Cells were shifted to 36°C for 4 h and then were treated with 30 J/m^2 UV-C or left untreated. Cell septation was then monitored by microscopy following a shift back to 25°C. (**B**) The percentage of cells entering mitosis for each indicated strain following the time course described in (**A**). Wild-type cells show a delay in mitotic entry following UV-C treatment while *chk1*Δ cells show no delay. The cell-cycle delay and checkpoint arrest of the *rad2*Δ *exo1-so* double mutant both before and after UV-C treatment are dependent on *ast1*. Graphs show the mean ± standard error ($n = 3$) for each data point. (**C**) Cultures of the indicated strains were grown at 30°C. The percentage of aberrant mitoses or 'cut' cells was monitored by microscopy both before and after the addition of thiamine. Graph shows the mean ± s.d. ($n = 3$) for each data point. The cell-cycle arrest of the *rad2*Δ *exo1-so* strain is dependent on *chk1* and partially dependent on *ast1*. The triple mutant *rad2*Δ *exo1-so chk1-S345A* shows an intermediate percentage of 'cut' cells following thiamine addition revealing a partial dependence on the phosphorylation of Chk1 on Serine 345 following DNA damage.

Rad2, Exo1 and Ast1 are critical for damage resistance in the absence of MRN function, but Ast1 is not as critical when the DNA ends are exposed by deletion of *pku70*.

XPG-related nucleases are required for recombination

The resection of lesions is not only required for checkpoint signalling, but also to form the structures required for HR-mediated repair of DSBs. Using an inducible system to generate a DSB with HO endonuclease (Outwin *et al*, 2009), we assayed for the recruitment of Ast1, Exo1 and Rad2 to this lesion by chromatin immunoprecipitation (ChIP). For this, we used RPA and Rad52 as positive controls as both are rapidly recruited to DSBs. Consistent with a role in DSB resection, each nuclease was recruited to both sides of the DSB (Figure 6A). As DSB formation in this system is limited to ~20% of chromatids, these data are actually a 5-fold underestimate of the DSB-induced recruitment of each of these proteins.

In order for HR to proceed, two ssDNA-binding proteins must be recruited to the resected DSBs in a sequential manner. First, the high affinity ssDNA-binding protein RPA is recruited. Subsequently, Rad52 catalyses the replacement of RPA with the Rad51 recombinase. To assess the ability of our double and triple nuclease mutant strains to perform HR, we assayed the appearance of these proteins in UV-C- and Bleomycin-induced foci in G2 arrested wild-type, *rad2*Δ

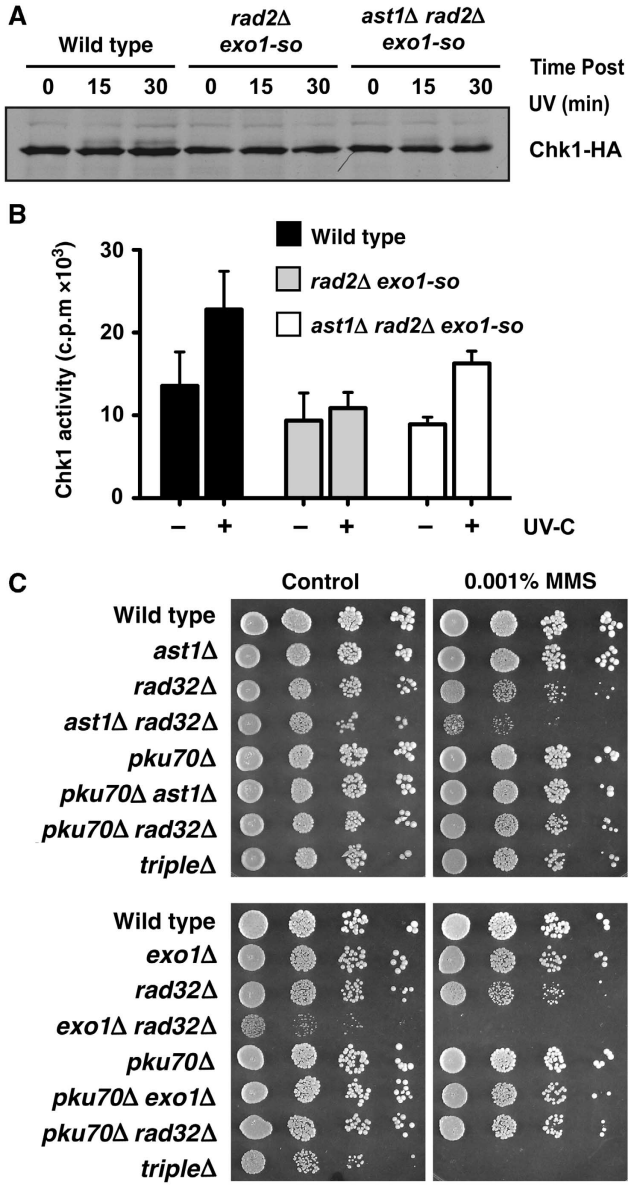


Figure 5 Multiple pathways are necessary to activate the DNA damage checkpoint. (**A**) Western blot with anti-HA antibody and extracts from the indicated strains containing HA-tagged *chk1*. Samples were taken at the indicated times following the time course in Figure 4. The wild-type strain shows Chk1 activation following UV treatment while the *rad2*Δ *exo1-so* strain shows no Chk1 activation. The *ast1*Δ *rad2*Δ *exo1-so* strain has an intermediate level of Chk1 activation. (**B**) Chk1 kinase assay performed on the indicated strains containing HA-tagged *chk1*. Graph shows the mean ± s.d. for each data point ($n = 3$). The wild-type strain shows an increase in Chk1 activation following UV treatment while the *rad2*Δ *exo1-so* strain shows no increase in Chk1 activation above basal levels. The triple mutant *ast1*Δ *rad2*Δ *exo1-so* has an intermediate amount of Chk1 activation following UV treatment. (**C**) Ten-fold serial dilutions of the indicated strains were plated on media containing 0.001 % MMS or a control plate. Note that *pku70*Δ suppresses the increased MMS sensitivity of *rad32*Δ *ast1*Δ cells, but not *rad32*Δ *exo1*Δ cells, however, *pku70*Δ does suppress the slow growth phenotype of *rad32*Δ *exo1*Δ cells.

exo1-so and *ast1*Δ *rad2*Δ *exo1-so* strains. At the doses used, UV-C rapidly induces a range of lesions including substrates for both excision and recombinational repair (Callegari and Kelly, 2007). Bleomycin, a metabolite of *Streptomyces*, induces DSBs following a period of incubation. In each case, the percent of cells with foci containing these ssDNA-binding proteins was reduced in both double and triple mutants following DNA damage as compared to wild-type controls. While UV irradiation induced multiple Rad51 and RPA foci in wild-type cells, only single or occasionally two foci were visible in the positive double and triple mutants (Supplementary Figure 3). This may reflect less efficient ssDNA production in these strains, though DSBs can cluster (Lisby *et al*, 2003) and so a focus may represent more than one lesion. We noted that RPA (but not Rad51) foci were more prevalent in UV-C-irradiated cells than in Bleomycin-treated cells, presumably forming at lesions repaired by excision pathways that do not require Rad51 for repair (Figure 6B).

These data predict that the nuclease mutants might be defective in HR-mediated repair. We assayed this using tandemly duplicated heteroalleles of *ade6* flanking a *his3* gene (Osman *et al*, 1996). In this system, recombination can occur between chromatids, leaving the *his3* gene intact. However, in the absence of Rad51, this mode of recombination cannot occur, and a second Rad52-dependent recombination pathway know as single-stranded annealing (SSA) actually generates more recombinants due to the defect in error-free HR. In this scenario, 5′→3′ resection of the 8.5-kb intervening region, annealing of complementary *ade6* sequences and removal of ssDNA flaps can generate wild-type *ade6* alleles but results in the loss of the *his3* gene (Doe *et al*, 2004). Using this assay, both *exo1*Δ *rad51*Δ and *ast1*Δ *rad51*Δ cells had 65–70% fewer recombinants than a *rad51*Δ strain (Figure 6C). We could not assay *rad2*Δ in this system as it is synthetically lethal with *rad51*Δ (Murray *et al*, 1994), and similarly we have also failed to recover viable *ast1*Δ *exo1*Δ *rad51*Δ strains from extensive tetrad dissections. Together, these data show that the XP-G-related nucleases function in SSA-mediated recombination, and as flap removal is catalysed by the Rad1/10 nuclease (Fishman-Lobell and Haber, 1992; Ivanov and Haber, 1995; Toh *et al*, 2010), this is presumably through the 5′→3′ resection between repeats.

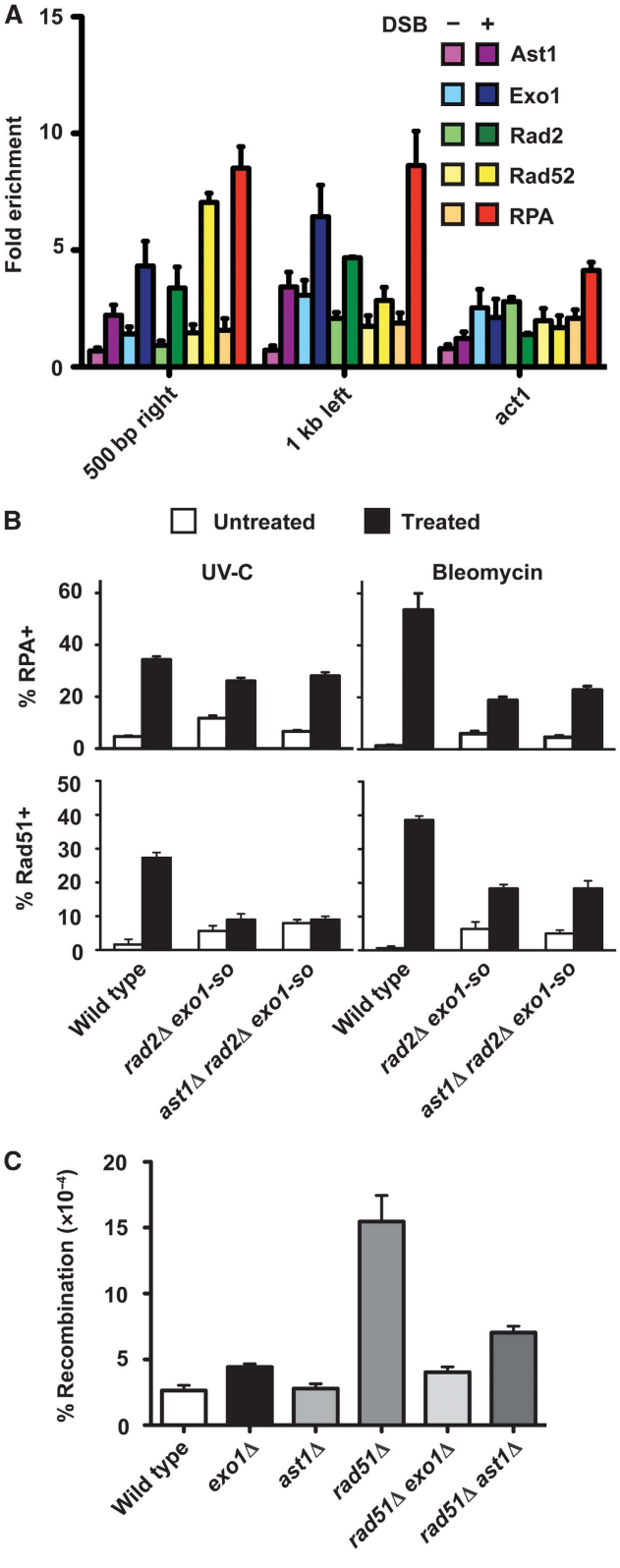
XPG-related nucleases are required for ssDNA production in vivo

The data thus far point to a requirement for Rad2 and Exo1 in the production of ssDNA following DNA damage in G2 cells. To assay this, we utilized high doses of UV-C to rapidly induce lesions in G2 synchronized cells. Subsequently, genomic DNA was extracted, and then processed by two Southern blotting methods (Figure 7A). First, a conventional Southern blot was employed, where an alkali transfer process was used to denature genomic DNA. Next, non-denatured DNA in a duplicate gel was transferred under native conditions, such that the only probe accessible ssDNA was produced in the cell. We used G2 synchronized cells for this assay in order to ensure that ssDNA was not produced through the unwinding of replication forks. For sensitivity, we used a probe to the 4.5-kb *Kpn*I-*Xho*I fragment of the non-transcribed spacer (NTS) region of the rDNA, which is present in ∼150 copies (Ampatzidou *et al*, 2006). In wild-type cells, UV-C treatment induced the production of ssDNA, resulting in a hybridization signal both above and below 4.5 kb that was ∼3.5-fold over background. This indicated resection had extended, at least in some cases, beyond the restriction sites. Conversely, and consistent with the Chk1 activity seen in these strains

(Figure 5), ssDNA production was ablated in *rad2*Δ *exo1-so* cells and significantly attenuated, almost to unirradiated levels, in *ast1*Δ *rad2*Δ *exo1-so* cells.

As a second assay for DSB end resection, we utilized a very recently developed system for Tet-regulated expression of the homing endonuclease I-PpoI in *S. pombe*, which efficiently induces one DSB in the 28S rDNA gene of each rDNA repeat (Sunder *et al*, 2012). We then performed qPCR on genomic DNA that had been extracted from the strains and digested with a panel of restriction endonucleases surrounding the I-PpoI cleavage site (Figure 7B). The production of ssDNA in this region ablates the restriction site that would otherwise interrupt the qPCR template (Zierhut and Diffley, 2008). Resection occurred at the induced DSB in wild-type cells, however, this resection was significantly more efficient if it initiated 3′ to the DSB, that is, in the direction of rDNA transcription. Consistent with the UV-induced resection results described above, the *rad2*Δ *exo1-so* double mutant showed background levels of resection, while the *ast1*Δ *rad2*Δ *exo1-so* triple mutant showed substantially attenuated resection close to the DSB.

Together, the data from these two assays confirm a requirement for Exo1 and Rad2 in the production of ssDNA induced by exogenous DNA damage, and is fully consistent with the reduced formation of RPA and Rad51 foci, defects in recombination, and the defective checkpoint responses in these strains. Curiously, the absence of *ast1* does not enhance these defects and actually slightly enhances ssDNA production and Chk1 activation in the *rad2*Δ *exo1-so* double mutant, suggesting that endogenous levels of Ast1 are critical mainly for endogenous damage in cells lacking Rad2 and Exo1.



Discussion

The purpose of this study was to identify factors, in an unbiased manner, that are capable of amplifying a DNA damage signal to Chk1, but do not bypass Chk1 function. Conventional loss-of-function genetic screens for checkpoint genes appear to have become saturated as no new genes have been identified by this protocol in recent years. Important regulators of the checkpoint response could still await identification though these factors, whether due to redundancy or other essential functions, would have escaped identification.

Our screen identified Ast1, a member of the XPG-related nuclease family that was yet to be functionally characterized. Our analysis of Ast1 function in combination with the other XPG-related family members, Rad2 and Exo1, shows that these proteins function in the processing of lesions into checkpoint- and HR-promoting ssDNA templates. This assertion is based on several findings: first, the checkpoint amplification and suppression of *chk1-E472D* by Ast1 overexpression; second, the HR defects of the single nuclease

Figure 6 Ast1, Exo1 and Rad2 play a role in DSB repair. (**A**) ChIP and qPCR of HA-Ast1, Myc-Exo1 and Myc-Rad2 strains before and after the induction of a site-specific DSB using an HO endonuclease. Graph shows the mean ± s.e.m. ($n = 3$–6) for each data point. RPA-GFP and Rad52-YFP are used as positive controls. Following induction of the break, all nucleases and positive controls are recruited to the DSB. A control primer, *act1*, was used for qPCR. Cutting efficiency for the inducible HO endonuclease in this system is ~20% of chromatids. (**B**) Quantification of RPA-GFP and Rad51 focus formation before and after treatment with 50 J/m^2 UV or 0.5 mU Bleomycin for the indicated strains by microscopy following the time course described in Figure 4. Graph shows the mean ± s.d. for each data point ($n = 3$). Fewer foci are seen in the *rad2*Δ *exo1-so* and *ast1*Δ *rad2*Δ *exo1-so* strains following UV and Bleomycin treatment than in the wild-type strain. (**C**) Quantification of the recombinants produced in a single-stranded annealing assay for the indicated strains. Both *ast1*Δ and *exo1*Δ single mutants had a reduced capacity for single-strand annealing as measured by this assay.

mutants; third, the checkpoint defects of double and triple nuclease mutants in response to intrinsic and exogenous DNA damage; and finally, the *in vivo* (RPA and Rad51 foci formation and nuclease localization to DSBs) and *ex vivo* (native Southern blotting following UV irradiation, and resection of I-PpoI-induced DSBs) assays showing defects in damage-induced ssDNA production in the absence of Rad2 and Exo1.

One curious but consistent observation was that adding *ast1*Δ to *rad2*Δ *exo1-so* actually leads to a modest but measurable increase in Chk1 activation in response to UV irradiation in G2 synchronized cells. This is in contrast to the spontaneous activation of Chk1 in *rad2*Δ and *exo1*Δ cells that presumably arise from endogenous replicative damage, and is diminished by *ast1*Δ (Figure 2D). In wild-type cells, Chk1 activation is largely stochastic; the duration of Chk1 activation, but not the actual activity, is proportional to the degree of DNA damage (Latif *et al*, 2004). Thus, the reduced Chk1 activation in the triple mutant must not have passed the threshold necessary for a checkpoint-induced delay, or alternatively may originate from only a small proportion of cells in the culture.

These are particularly unexpected results as in synchronous cultures, *ast1*Δ suppresses both an intrinsic and synthetic cell-cycle delay in *rad2*Δ *exo1-so* cells, and a further extended delay induced by UV irradiation of the double mutant, suggesting that more complex signalling is occurring in this context. We note that most DNA repair mutants continue to cycle with activated Chk1, that active Chk1 signalling is antagonized by the Cdr kinases via regulation of Wee1 (Calonge and O'Connell, 2006; Calonge *et al*, 2010), and that irradiated wild-type cells reactivate Chk1 after passage through mitosis without cell-cycle delay (den Elzen and O'Connell, 2004). Thus, Chk1 activity does not always correlate with cell-cycle progression.

However, the triple mutant also produces a small amount of ssDNA in response to DNA damage, which could be responsible for the low level Chk1 activation. At this stage, we do not know the source of this ssDNA, though we note that Ast1, with Rad2 and Exo1, is recruited to DSBs (Figure 6). While it seems to play an important role in response to endogenous lesions formed in the absence of Rad2 and Exo1, Ast1 may be inefficient at processing lesions in G2 resulting from exogenous DNA damage. Consistent with this, *ast1*Δ cells do not show G2 checkpoint defects, and the ability of Ast1 to amplify G2 checkpoint signalling in response to exogenous sources of DNA damage requires its overexpression. Therefore, deletion of *ast1* in the absence of Rad2 and Exo1 may enable other nucleases to localize to and resect DNA strand breaks.

Importantly, even though *ast1*Δ and *exo1*Δ are not hypersensitive to DNA damage, they greatly sensitize the MRN

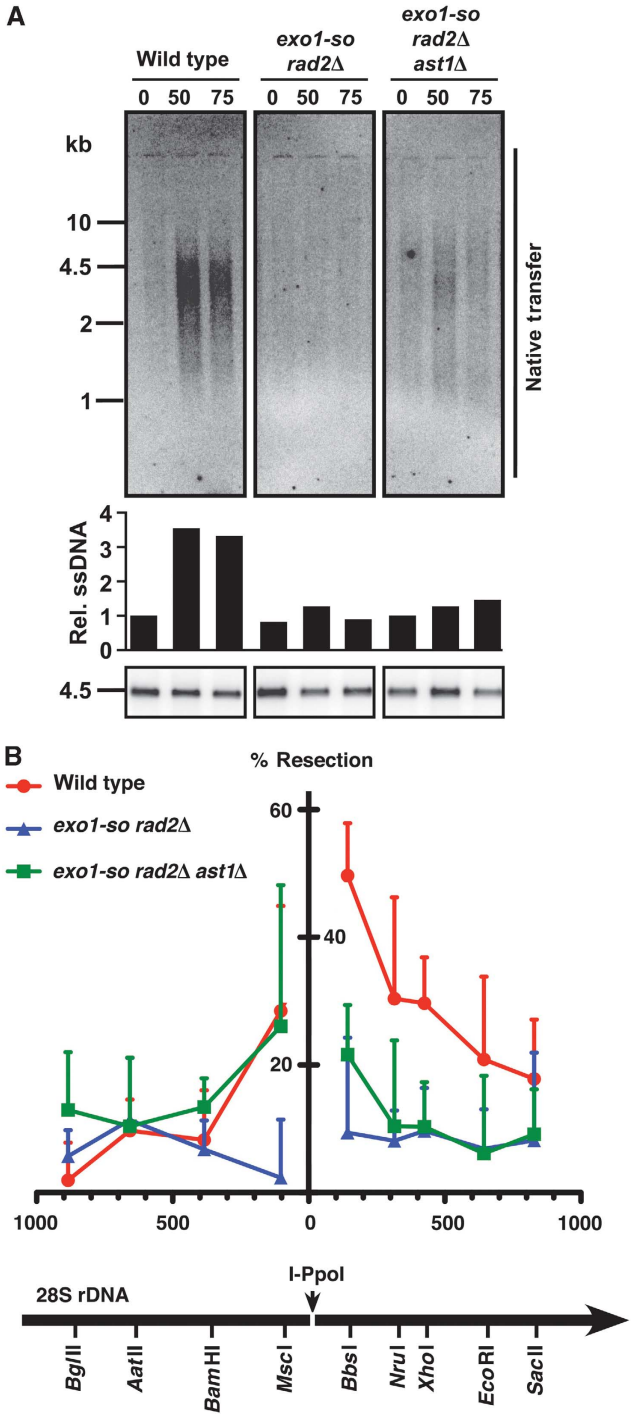


Figure 7 Cells lacking *ast1*, *exo1* and *rad2* have a defect in ssDNA production. (**A**) Non-denaturing Southern blot of the indicated strains treated with the indicated doses of UV following the time course described in Figure 4. The blot performed under native conditions (20 × SSC) is shown at the top of the figure while the denaturing Southern blot loading control is shown underneath. As seen in the native Southern blot, the wild-type strain had an increase in ssDNA production following UV treatment. The *rad2*Δ *exo1-so* strain did not have an increase in ssDNA production while the *ast1*Δ *rad2*Δ *exo1-so* strain had a small increase. The denaturing Southern blot shows a 4.5-kb band of the same intensity for all strains under all conditions as a loading control. The probe used for both Southern blots is an *Xho*I and *Kpn*I fragment that recognizes the non-transcribed spacer (NTS) region of the *S. pombe* rDNA locus. The quantification of signal is in arbitrary phosphorimager units, corrected to the loading control (the corresponding track in the denatured transfer), and normalized to unirradiated wild-type cells. (**B**) qPCR analysis of *in vivo* resection of an I-PpoI-induced DSB within the 28S gene of the rDNA repeats. Cutting efficiency in this system is ⩾90% of rDNA repeats. The restriction map shows the position of sites on each side of the I-PpoI site used in the analysis where numbers indicate nucleotides, and the arrow depicts the direction of transcription. Data are mean ± s.d. for three independent experiments.

mutant *rad32*Δ to MMS. As *pku70*Δ suppresses the MMS sensitivity of *rad32*Δ in an Exo1-dependent manner, it has been proposed that Ku blocks Exo1 from accessing DSBs (Tomita *et al*, 2003; Mimitou and Symington, 2010). Consistent with this notion, the increased sensitivity of *rad32*Δ *ast1*Δ cells is suppressed by *pku70*Δ, presumably by granting Exo1 access to DSB ends. Thus, in the absence of MRN, Exo1 and Ast1 have important but non-overlapping functions. Furthermore, the viability of *rad32*Δ is severely impaired by *exo1*Δ (Figure 5) and completely lost on a *rad2*Δ background (Tavassoli *et al*, 1995). However, unlike the enhanced MMS sensitivity, the slow growth of *rad32*Δ *exo1*Δ, which presumably originates in spontaneous DNA damage, is suppressed by *pku70*Δ. In this context, the absence of Ku must allow one or more of the other nucleases present in the cell to process endogenous lesions into checkpoint-signalling and repairable structures. Therefore, we propose that XPG-dependent processing is an alternative mechanism used to arrive at the same structures. Given that lesion processing is so critical to mount a DNA damage response, it is not surprising that there would be more than one mechanism responsible.

Each of these XPG-related nucleases is highly conserved from *S. pombe* to humans. Our experiments indicate that their function in initiating a response to DNA damage is significantly redundant. Why then would three independent enzymes be conserved through evolution? The pressure for this conservation may come from non-redundant affinities for specific DNA structures or DNA ends. Most studies of end resection in *S. cerevisiae* have utilized an HO-endonuclease induced DSB, which cleaves DNA to leave a 4-bp 3′-overhang (Kostriken *et al*, 1983; Kostriken and Heffron, 1984). The agents we have used to induce DNA damage in this study generate a number of structures containing strand breaks, either directly or as an intermediate during their repair. Clearly each of these nucleases participates in the cellular responses to these agents and therefore may recognize a diversity of structures. The development of tools to efficiently induce different specific lesions in a site-specific manner may facilitate the discovery of non-overlapping functions for these nucleases.

Selective pressure for conservation of Exo1 and Rad2 may also come from functions in Okazaki Fragment processing and excision repair pathways. What then provides selective pressure for conservation of Ast1? The fact that *ast1*Δ cells by themselves lack a strong phenotype does not rule out an important role in DNA metabolism, as the same criterion could be applied to *exo1*Δ. In addition to being required for checkpoint signalling generated by endogenous DNA damage in cells lacking Rad2 and Exo1 (Figures 2 and 4), Ast1 is required for the SSA pathway of recombination, and for damage resistance in the absence of the MRN complex (Figures 5 and 6). Nevertheless, the absence of a strong phenotype in *ast1*Δ cells negates the use of pathway interrogation by epistasis analysis, which has proven extremely useful in a plethora of DNA damage-sensitive mutants. We have crossed *ast1*Δ to a number of mutants defective in replication fork stability, damage tolerance, error-free HR, and excision repair pathways, and have yet to see a modification of phenotype, nor have we found a DNA damaging agent to which to *ast1*Δ cells are hypersensitive. Further, we have no evidence for meiotic defects in *ast1*Δ cells. Thus, although a unique function for Ast1 has so far eluded identification, the conservation of this enzyme over vast evolutionary distance argues that such a function must exist, in addition to any functions that are overlapping with Exo1 and Rad2.

An important aspect of this study is that we have been able to separate the function of these nucleases in G2 phase from their other functions that are critical during DNA replication. This is not a unique scenario as dual functions in replication and post-replicative DNA damage responses have been described for the mediator protein Cut5/TopBP1 (Araki *et al*, 1995; McFarlane *et al*, 1997; Verkade and O'Connell, 1998; Makiniemi *et al*, 2001), and for Replication Factor C (Kim and Brill, 2001; Bermudez *et al*, 2003). Although *S. cerevisiae* does not have an Ast1 homologue, any checkpoint defect for *rad27*Δ *exo1*Δ double mutants would also have to be assayed conditionally and separated from the defect in Okazaki fragment processing that is the probable cause of the lethality in this strain (Tishkoff *et al*, 1997). All three nucleases are conserved in human cells, and this very conservation argues that the human nucleases would possess the same functions as the fission yeast enzymes. However, a demonstration of this would also require separation of a checkpoint function from the repair and replication functions that operate during S phase.

Our screen was designed to take into account both genetic redundancy and diversity of function (including essential functions) that prevents gene identification in loss-of-function screens. It is therefore possible, if not likely, that additional mechanisms to initiate a DNA damage response may exist and have yet to be identified. We have previously found that various structure specific nucleases assigned to particular DNA repair pathways actually participate in diverse DNA damage responses (Sheedy *et al*, 2005; Lee *et al*, 2007). Conceivably, these nucleases could be checkpoint-initiating enzymes, though no nucleases other than Ast1 came out of our screen. Therefore, the execution of additional screening strategies seems warranted and likely to uncover additional novel components and pathways that control the cellular response to DNA damage.

Materials and methods

General S. pombe methods

The strains used for experiments were all derived from $972h^-$ and $975h^+$. Standard genetic procedures were used as described previously (Moreno *et al*, 1991). Crosses were performed by tetrad and random spore analysis and multiple isolates of each resulting strain were tested. 13 × Myc-tagged alleles of *exo1* and *rad2* were constructed using targeting constructs to the 3′ end of the open reading frame of each gene. A tagged allele of *ast1* was created by tagging a genomic clone of *ast1* with a triple-HA tag including a *his3* marker and integrating it into the *ast1* genomic locus. The functionality of each tagged allele was confirmed by testing for resistance to MMS. Methods for detection of proteins by microcopy and western blotting were as described (Outwin *et al*, 2009; Tapia-Alveal and O'Connell, 2011). In order to shut-off expression of Exo1 to create an *exo1 ast1 rad2* conditional triple mutant, an *exo1* allele under the control of the weakest thiamine repressible promoter (*nmt1*) was integrated into the Exo1 genomic locus. Chk1 activity was assayed in triplicate from anti-HA IPs as described (den Elzen and O'Connell, 2004). Single-strand annealing assays were performed as described in Osman *et al* (1996) where the strains of interest were crossed into a background strain containing the $his3^+$ gene flanked by two $ade6^-$ heteroalleles. To measure *exo1* expression levels, total RNA was extracted with Trizol reagent (Invitrogen), reverse transcribed into cDNA with Quanta Bio

qScript cDNA Supermix, and mRNA levels determined by q-RT-PCR normalized to *act1*.

Multicopy genomic plasmid screen

A multicopy plasmid screen using a genomic library cloned into the vector pURSP1 (Barbet *et al*, 1992) was used to isolate genes that when present in multiple copies suppressed the synthetic lethality between a temperature-sensitive allele of *chk1* (*chk1-E472D*) and *cdc27-P11* at 32.5°C. Plasmids from suppressors that grew at 32.5°C but not at 36°C were recovered and then tested for their ability to suppress the *chk1-E472D* sensitivity to MMS at 36°C. Ast1 was identified six independent times in this screen. A nuclease-dead derivative of Ast1 was generated by mutating catalytic Aspartates (D158, 160 179 and 181) to Alanines by the methods of Kunkel *et al* (1987) using the oligonucleotides listed in Supplementary Table 1.

cdc25-22 block and release time course with UV irradiation

Cells were grown to logarithmic phase in supplemented minimal medium at 25°C. Cells were then shifted to 36°C to arrest them in G2 for 4 h. For experiments with *exo1-so*, thiamine was added to 10 μM 2 h prior to shift to 36°C. Following this arrest, 25–50 ml of cells was collected by filtration (3–4 filters per 50 ml, 2 filters per 25 ml) and the filters containing the cells were irradiated with the indicated dose of UV (30 or 50 J/m^2). The irradiated filters were added to new supplemented minimal medium and samples of 1 ml were fixed with 70% ethanol every 15 min for 3 h. For western blotting, Southern blotting or microscopy, cultures were allowed to recover for 30 min following UV irradiation and samples were fixed as described for each experiment.

Southern blotting

Genomic DNA was extracted from irradiated or unirradiated exponentially growing cells, digested with *Kpn*I and *Xho*I, and separated on duplicate Agarose gels. Southern blotting was performed to Zeta-Probe membrane (BioRad). One gel was transferred with 0.4 M NaOH, the standard transfer condition, which denatures all DNA. The duplicate gel was transferred in 20× SSC without prior treatment, and DNA crosslinked to the membrane with a Stratalinker (Stratagene). Both membranes were hybridized under standard conditions using the 4.5-kb *Kpn*I-*Xho*I fragment of the rDNA NTS region (Ampatzidou *et al*, 2006). Hybridization signals were quantified on a BioRad FX phosphorimager. ssDNA production was quantified from the 20× SSC transfer and normalized to the NaOH transfer.

Generation and analysis of site-specific DSBs

For ChIP analysis, an HO endonuclease cleavage site was generated within a *MATa* site integrated at the *his3* locus as previously described (Outwin *et al*, 2009). Briefly, a temperature-sensitive allele of HO was expressed from the *nmt1* promoter at 36°C (HO inactive), and following shift to 25°C for 2 h, HO is activated and induces a DSB in ~20% of chromatids. At this time point, cells were fixed in formaldehyde and process for ChIP analysis as described (Outwin *et al*, 2009; Tapia-Alveal and O'Connell, 2011) using antibodies to HA, Myc or YFP/GFP epitopes, and qPCR primers (Supplementary Table 2). Experiments are performed in triplicate with 3–6 biological replicates.

For I-PpoI induced DSBs in the rDNA, plasmid pSS16 encoding both the Tet-regulator from the *adh1* promoter, and the I-PpoI endonuclease from a Tet-regulated Cauliflower Mosaic Virus 35S promoter (Sunder *et al*, 2012), was linearized with *Not*I and integrated at the *arg3* locus in wild-type, *rad2*Δ *exo1-so*, and *rad2*Δ *exo1-so ast1*Δ strains. Strains were propagated in EMM2 minimal media with 3.6 g/l sodium glutamate as the nitrogen source. In all, 10 μM thiamine was added to the cultures to repress *exo1-so*, and then 4 μM anhydrotetracycline (ahTET) was added for an additional 2 h to half the cultures. Cells were harvested by centrifugation, washed in ice-cold stop buffer (150 mM NaCl, 50 mM NaF, 10 mM EDTA, 1 mM NaN_3), and genomic DNA extracted (Moreno *et al*, 1991). The efficiency of DSB formation was assayed by qPCR using oligonucleotides 516 and 517 (Sunder *et al*, 2012) against a standard curve of wild-type DNA, and confirmed to occur at ~90% efficiency. Genomic DNA was then digested with *Bgl*II, *Aat*II, *Bam*HI, *Msc*I, *Bbs*I, *Nru*I, *Xho*I, *Eco*RI or *Sac*II, and processed by qPCR. The efficiency of *in vivo* resection was estimated by the ability of these restriction enzymes to ablate qPCR products. Cycle times (CT) were determined ± restriction enzyme to calculate a ΔCT, and remaining percentage of DNA remaining ± I-PpoI expression was calculated from the standard curve. The percent resection was calculated as the DNA remaining after I-PpoI digestion (+ ahTET) minus that without digestion (− ahTET), and then multiplied by 2 to account for the halving of DNA by resection. qPCR primers spanning each restriction site are listed in Supplementary Table 3.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Author contributions: KK performed the experiments with assistance by MOC. KK and MOC planned the study, analysed data and wrote the manuscript.

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