

# Nonhomologous end-joining promotes resistance to DNA damage in the absence of an ADP-ribosyltransferase that signals DNA single strand breaks

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## Summary

ADP-ribosylation of proteins at DNA lesions by ADP-ribosyltransferases (ARTs) is an early response to DNA damage. The best defined role of ADP-ribosylation in the DNA damage response is in repair of single strand breaks (SSBs). Recently, we initiated a study of how ADP-ribosylation regulates DNA repair in *Dictyostelium* and found that two ARTs (Adprt1b and Adprt2) are required for tolerance of cells to SSBs, and a third ART (Adprt1a) promotes nonhomologous end-joining (NHEJ). Here we report that disruption of *adprt2* results in accumulation of DNA damage throughout the cell cycle following exposure to agents that induce base damage and DNA SSBs. Although ADP-ribosylation is evident in *adprt2*<sup>-</sup> cells exposed to methylmethanesulfonate (MMS), disruption of *adprt1a* and *adprt2* in combination abolishes this response and further sensitises cells to this agent, indicating that in the absence of Adprt2, Adprt1a signals MMS-induced DNA lesions to promote resistance of cells to DNA damage. As a consequence of defective signalling of SSBs by Adprt2, Adprt1a is required to assemble NHEJ factors in chromatin, and disruption of the NHEJ pathway in combination with *adprt2* increases sensitivity of cells to MMS. Taken together, these data indicate overlapping functions of different ARTs in signalling DNA damage, and illustrate a critical requirement for NHEJ in maintaining cell viability in the absence of an effective SSB response.

**Key words:** *Dictyostelium*, ADP-ribosyltransferases, PARPs, Nonhomologous end-joining

## Introduction

ADP-ribosylation is the addition of single or multiple ADP-ribose moieties to target proteins (D'Amours et al., 1999). ADP-ribosyltransferases (ARTs), the enzymes that catalyse this post-translational modification, are found in a variety of eukaryotes, with 22 genes containing a predicted ART catalytic domain identified in humans (Hottiger et al., 2010). ADP-ribosylation has been implicated in regulating a variety of cellular processes including DNA repair, cell growth and differentiation, transcriptional regulation and programmed cell death (Hottiger et al., 2010; Messner and Hottiger, 2011; Quénet et al., 2009).

The best defined role of ARTs is in the cellular response to DNA damage, with particular reference to DNA strand breaks. PARP1, the founding member of the ART family, is recruited to single strand breaks (SSBs), becomes activated, and modifies a number of proteins at DNA lesions, including itself (Caldecott, 2008; Krishnakumar and Kraus, 2010). Resolution of SSBs is reduced in the presence of ART inhibitors (ARTi), or following depletion of PARP1 (Ding et al., 1992; Durkacz et al., 1980; Fisher et al., 2007; Morgan and Cleaver, 1983; Schraufstatter et al., 1986). Similarly, mice disrupted in the *parp1* gene are sensitive to DNA damaging agents that induce base damage or strand breakage and exhibit delayed kinetics of SSB repair (SSBR) (de Murcia et al., 1997; Le Page et al., 2003; Masutani

et al., 1999; Trucco et al., 1998; Trucco et al., 1999). More recently, a second ART (PARP2) has been implicated in SSBR. Similar to *parp1*<sup>-/-</sup> cells, *parp2*<sup>-/-</sup> mice are sensitive to DNA damage, exhibit increased chromosome instability and a delay in repair following exposure to alkylating agents (Ménissier de Murcia et al., 2003; Schreiber et al., 2002). While the precise mechanism by which ARTs regulate SSBR remains to be defined, ADP-ribosylation of target proteins, including histones, is thought to facilitate chromatin relaxation around the break and to recruit chromatin remodelling enzymes to DNA lesions (Ahel et al., 2009; Gottschalk et al., 2009; Mehrotra et al., 2011; Timinszky et al., 2009). An additional non-mutually exclusive function of ADP-ribosylation is to enrich repair factors at DNA lesions through poly-ADP ribose (PAR) interaction domains located in these proteins (Bekker-Jensen et al., 2007; Caldecott et al., 1996; El-Khamisy et al., 2003; Iles et al., 2007; Kanno et al., 2007; Masson et al., 1998; Okano et al., 2003; Rulten et al., 2008).

In addition to regulating SSBR, ARTs are also required for resolution of DNA double strand breaks (DSBs). ARTi sensitise mammalian cells to DSBs (Audebert et al., 2004; Boulton et al., 1999) and delay repair of this variety of DNA lesion (Rulten et al., 2011). DNA DSBs can be repaired by homologous recombination (HR) or nonhomologous end-joining (NHEJ) (Branzei and

Foiani, 2008). PARP1 has been implicated in HR-mediated repair of DSBs at stalled and/or damaged replication forks (Bryant et al., 2009; Sugimura et al., 2008; Yang et al., 2004), in addition to alternative NHEJ (A-NHEJ), an end-joining pathway that is activated in the absence of core NHEJ factors such as DNA-PKcs and Ku (Audebert et al., 2004; Brown et al., 2002; Robert et al., 2009; Wang et al., 2006). However, its role in classic NHEJ is less clear. While PARP1 interacts with Ku and DNA-PKcs (Ariumi et al., 1999; Galande and Kohwi-Shigematsu, 1999; Li et al., 2004; Ruscetti et al., 1998), classic NHEJ is normal in murine *parp1*<sup>-/-</sup> cells (Yang et al., 2004), and depletion of PARP1 or PARP2 has little impact on DSB resolution (Rulten et al., 2011). Instead, we and others recently identified a third ART (PARP3/Adprt1a) that becomes activated in response to DNA DSBs and promotes classic NHEJ by facilitating enrichment of NHEJ factors at DNA lesions through PAR-interaction domains located in these proteins (Boehler et al., 2011; Couto et al., 2011; Loseva et al., 2010; Rulten et al., 2011).

Taken together these studies indicate that different ARTs respond to different varieties of DNA damage. However, given that ARTs have been implicated in regulating multiple DNA repair pathways, it is possible redundancy exists between these enzymes to allow cells to tolerate genotoxic stress. For example, reduced ADP-ribosylation is apparent in *parp1*<sup>-/-</sup> cells (Shieh et al., 1998) and resolution of DNA strand breaks is only delayed in these cells (Le Page et al., 2003; Trucco et al., 1998). Further, disruption of murine PARP1 and PARP2 in combination results in embryonic lethality indicating redundancy between different ARTs during the development process and/or in maintaining genome stability (Ménissier de Murcia et al., 2003). Recently, ARTs have received much attention as therapeutic agents that specifically kill tumours defective in HR due to the inability of these cells to resolve unrepaired SSBs that are converted to DSBs during DNA replication (Bryant et al., 2005; Farmer et al., 2005; Mendes-Pereira et al., 2009; Sourisseau et al., 2010; Weston et al., 2010; Williamson et al., 2010). However, these agents inhibit multiple ARTs, including PARP1–3 (Loseva et al., 2010). Therefore, assessing how different ARTs regulate compensatory repair mechanisms following genotoxic stress will further rationalise the use of ARTs in the clinic.

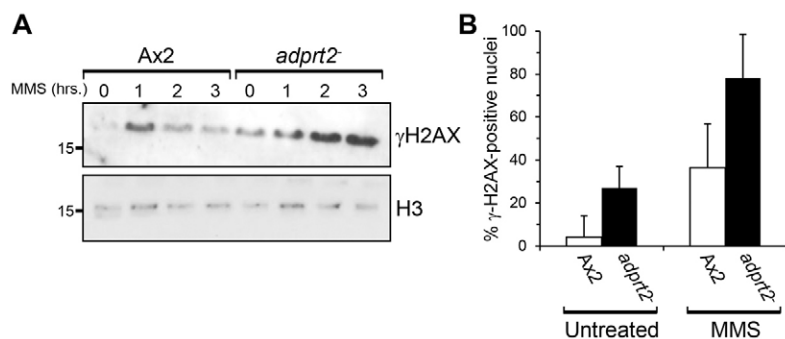
Recently, we and others initiated a study of DNA repair in *Dictyostelium* and found it contains certain DNA repair proteins absent in other invertebrates (Block and Lees-Miller, 2005; Hsu et al., 2006; Hudson et al., 2005; Zhang et al., 2009). For example, while poly-ADP-ribosylation is absent in yeast, ART activity is evident in *Dictyostelium* and ADP-ribosyltransferases (Adprts) have been isolated from this organism (Kofler et al.,

1993; Rickwood and Osman, 1979). Similar to vertebrates, two *Dictyostelium* ARTs (Adprt1b and Adprt2) promote resistance of cells to agents that induce SSBs (Couto et al., 2011; Pears et al., 2012). Further, we identified an additional ART (Adprt1a) that although dispensable for tolerance of cells to SSBs, promotes NHEJ through enhancing accumulation of Ku at DNA lesions (Couto et al., 2011; Pears et al., 2012). Taken together, these observations suggest *Dictyostelium* will prove an important model to assess certain DNA repair pathways, including those that employ ARTs. Here we exploit the genetic tractability of *Dictyostelium* to uncover novel cross-talk between ARTs following genotoxic stress. We find that disruption of the SSB-responsive ART Adprt2 results in accumulation of DNA damage and that this is signalled by the DSB-responsive ART Adprt1a. Further, we identify that Adprt1a-dependent signalling of MMS-induced DNA lesions channels repair through NHEJ and that this is required for cells to tolerate base damage or SSBs in the absence of Adprt2.

## Results

### Disruption of the *adprt2* gene results in increased frequency of DNA damage

Previously, we identified that two ARTs (Adprt2 and Adprt1b) are required for cells to tolerate base damage induced by methylmethanesulfonate (MMS) (Couto et al., 2011). Therefore, we wished to investigate the impact of disrupting *adprt2* on chromosome stability either in the absence or presence of this genotoxic agent. Accordingly, *adprt2*<sup>-/-</sup> cells were left untreated or exposed to MMS and accumulation of DNA damage established by isolating chromatin fractions from cells and assessing the levels of phosphorylated H2AX ( $\gamma$ H2AX) by western blotting. While little H2AX phosphorylation is observed in the absence of genotoxic stress in chromatin fractions prepared from parental Ax2 cells, moderate induction of  $\gamma$ H2AX is observed following administration of MMS (Fig. 1A). In contrast to its parent strain, slightly elevated levels of  $\gamma$ H2AX are observed in *adprt2*<sup>-/-</sup> chromatin fractions in the absence of genotoxic agents, with robust induction of  $\gamma$ H2AX apparent following exposure of these cells to MMS (Fig. 1A). Given that formation of DNA-damage-induced nuclear foci is a commonly used marker for post-translational modifications at or adjacent to sites of DNA damage, we additionally assessed accumulation of  $\gamma$ H2AX nuclear foci following exposure of cells to MMS. While low levels of  $\gamma$ H2AX-positive nuclei are apparent in Ax2 cultures in untreated cells, this is increased slightly upon exposure to MMS (Fig. 1B). In contrast, *adprt2*<sup>-/-</sup> cells exhibit elevated numbers of  $\gamma$ H2AX-positive nuclei in the absence of genotoxic stress



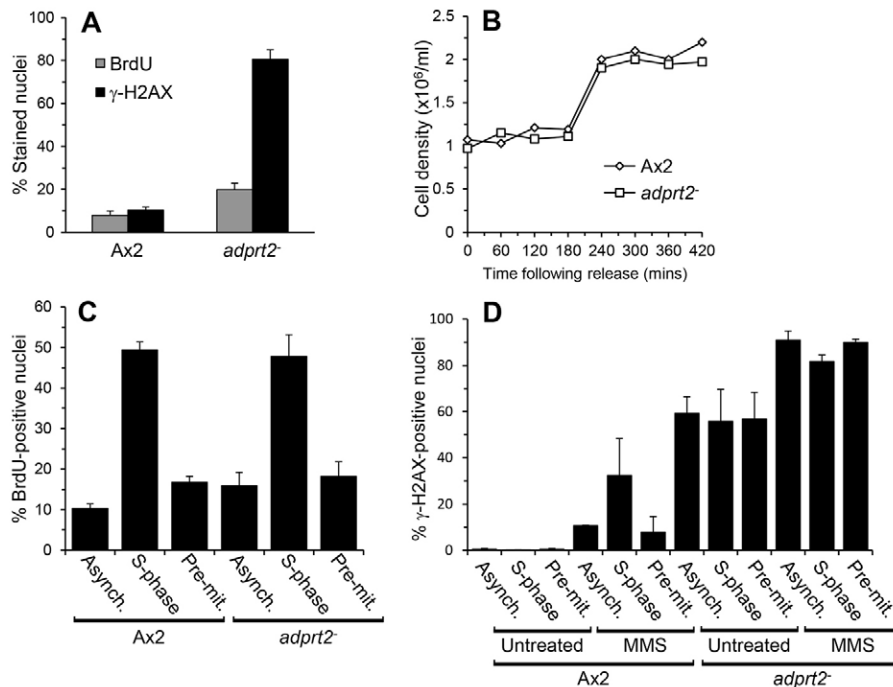
**Fig. 1. Loss of Adprt2 results in increased phosphorylation of H2AX in response to MMS.** (A) Ax2 and *adprt2*<sup>-/-</sup> cells were mock-treated or exposed to 5 mM MMS for the indicated times. Following subcellular fractionation, chromatin-enriched fractions were analysed by western blotting using the antibodies as indicated. (B) Ax2 and *adprt2*<sup>-/-</sup> cells were left untreated or exposed 5 mM MMS for 30 minutes. Cells were subjected to immunofluorescence using  $\gamma$ H2AX antibodies. The percentage of  $\gamma$ H2AX-positive cells was scored from a population of >200 cells. Error bars indicate the standard error of the mean (s.e.m.) from three independent experiments.

compared to Ax2 and this is further increased following exposure to MMS. Taken together, these data illustrate that loss of *Adprt2* results in accumulation of DNA damage in the absence of genotoxic stress that is further exacerbated upon exposure to agents that induce base damage and DNA SSBs.

Defective SSBR results in unrepaired SSBs that can be converted to DSBs as replication forks encounter DNA lesions during S-phase. Therefore, a possible explanation for the increased  $\gamma$ H2AX staining in *adprt2*<sup>-</sup> cells is that additional DNA damage is sustained during DNA replication. To test this possibility, Ax2 and *adprt2*<sup>-</sup> cells were exposed to MMS and subjected to staining with antibodies that recognise  $\gamma$ H2AX. In parallel, cells were left untreated and incubated with bromodeoxyuridine (BrdU) to mark cells undergoing S-phase. Cells positive for  $\gamma$ H2AX or BrdU were scored to allow comparison of the number of cells that sustain DNA damage with those undergoing S-phase at the time of MMS administration. The numbers of BrdU- and  $\gamma$ H2AX-positive nuclei are similar in Ax2 cultures (Fig. 2A). In contrast, while 19.8 $\pm$ 3% of *adprt2*<sup>-</sup> cells are undergoing DNA replication at the time of MMS treatment, 80.5 $\pm$ 4.6% are positive for  $\gamma$ H2AX following administration of DNA damage (Fig. 2A). The larger proportion of cells that exhibit  $\gamma$ H2AX staining above those that

are replicating at the time of MMS treatment indicates that while disruption of *adprt2* results in accumulation of DNA damage, this is not restricted to S-phase, but can accumulate at other stages of the cell cycle.

The number of  $\gamma$ H2AX-positive Ax2 cells is similar to those undergoing DNA replication at the time of exposure to MMS (10.32 $\pm$ 1.5 and 7.5 $\pm$ 2% respectively; Fig. 2A). An interpretation of these data are that in contrast to *adprt2*<sup>-</sup> cells, Ax2 cells accumulate damage during DNA replication following exposure to MMS. To assess this possibility, we induced Ax2 and *adprt2*<sup>-</sup> cells to synchronously pass through the cell cycle following release from growth arrest induced by cold shock (Araki et al., 1994), and administered DNA damage to cell populations enriched in S-phase. Release of Ax2 and *adprt2*<sup>-</sup> cells from cold shock induces synchronous cell division between 2 and 4 hours following temperature shift (Fig. 2B). *Dictyostelium* have no discernible G1, a short S-phase and protracted G2 (Muramoto and Chubb, 2008; Weijer et al., 1984). Accordingly, we predict that immediately following cell division cells will enter S-phase. Consistent with this and previous reports (Araki et al., 1994; MacWilliams et al., 2006; Strasser et al., 2012), we observe increased numbers of BrdU-stained cells immediately following cell division between 3 and 4 hours after



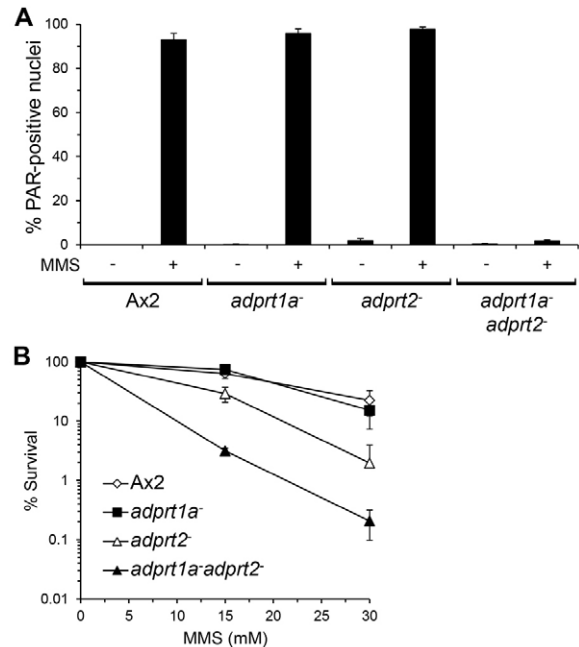
**Fig. 2. MMS induces elevated levels of DNA damage throughout the cell cycle in *adprt2*<sup>-</sup> cells.** (A) Following exposure to 1 mM MMS for 30 minutes, Ax2 and *adprt2*<sup>-</sup> cells were stained with antibodies that recognise phosphorylated H2AX ( $\gamma$ H2AX). In parallel, cells were left untreated and incubated in 100  $\mu$ M BrdU for 30 minutes and subsequently subjected to immunofluorescence using antibodies against BrdU. The percentages of  $\gamma$ H2AX- and BrdU-positive cells were scored from a population of >200 cells. Error bars indicate the s.e.m. from three independent experiments. (B) Cell cycle arrest was induced in Ax2 and *adprt2*<sup>-</sup> cells by incubating at 9.5°C for 16 hours. Subsequently, cells were released from this block by raising the temperature of the cultures to 22°C, allowing them to pass synchronously through the cell cycle. Cell density was monitored every hour post-release for both strains. Data are representative of three independent experiments. (C) Asynchronously growing Ax2 and *adprt2*<sup>-</sup> cells (Asynch.), or synchronised Ax2 and *adprt2*<sup>-</sup> cells taken at times corresponding to points at which cell populations are undergoing DNA replication (S-phase) or prior to the second mitotic division (pre-mit.), were incubated with 100  $\mu$ M BrdU for 30 minutes to label S-phase cells. Cells were then processed for immunofluorescence using an antibody against BrdU. At least 200 nuclei were assessed per condition to determine the percentage of BrdU-positive cells. Error bars indicate the s.e.m. from three independent experiments. (D) Following exposure to MMS for 30 minutes, asynchronous, S-phase-enriched or pre-mitotic Ax2 and *adprt2*<sup>-</sup> cell populations were subjected to immunofluorescence using an antibody raised against  $\gamma$ H2AX. The percentage of  $\gamma$ H2AX-positive cells was scored from a population of >200 nuclei. Error bars indicate the s.e.m. from three independent experiments.

release from cell cycle arrest (data not shown and Fig. 2C). The number of BrdU-positive cells is subsequently reduced to levels comparable with asynchronous cell cultures 6–7 hours following release from cold shock and prior to the second synchronous cell division. The percentage of *adprt2*<sup>-</sup> cells that exhibit nuclear  $\gamma$ H2AX staining following exposure to MMS is not significantly different in asynchronous, S-phase, or pre-mitotic cultures. The absence of a further increase in the number of nuclei that exhibit MMS-induced H2AX phosphorylation in S-phase-enriched cultures further supports the hypothesis that DNA damage is not restricted to S-phase in *adprt2*<sup>-</sup> cells (Fig. 2D). In contrast, while asynchronous Ax2 cells exhibit relatively low levels of nuclear  $\gamma$ H2AX staining following exposure to MMS, this is increased in S-phase-enriched cultures, with a subsequent reduction in pre-mitotic cells. In all instances, the percentage of Ax2 nuclei that exhibit  $\gamma$ H2AX staining does not differ significantly from those undergoing S-phase ( $P > 0.1$ ). The similar profiles of cells that accumulate  $\gamma$ H2AX with those undergoing DNA replication suggests that DNA damage occurs predominantly during S-phase in these cells. Taken together, we conclude that while MMS induces DNA damage during S-phase in Ax2 cells, disruption of *adprt2* renders cells more susceptible to accumulating DNA damage throughout the cell cycle.

#### Adprt1a signals MMS-induced DNA lesions in the absence of Adprt2

Although *adprt2*<sup>-</sup> cells are sensitive to agents that induce SSBs, ADP-ribosylation is evident in these cells (Couto et al., 2011). These data indicate that DNA damage observed following administration of MMS to *adprt2*<sup>-</sup> cells is signalled by ARTs other than Adprt2. In addition to Adprt2, Adprt1b and Adprt1a also function in signalling DNA damage in *Dictyostelium* cells (Couto et al., 2011). To test whether either of these proteins is responsible for the observed ADP-ribosylation in *adprt2*<sup>-</sup> cells, we attempted to disrupt *adprt1b* or *adprt1a* in combination with *adprt2*. Despite repeated attempts, we have been unable to disrupt *adprt2* and *adprt1b* in combination. In contrast, we were successful in generating an *adprt1a*<sup>-</sup>*adprt2*<sup>-</sup> strain. Ax2, *adprt1a*<sup>-</sup>*adprt2*<sup>-</sup> or the relevant single mutants were exposed to MMS and the ability of cells to ADP-ribosylate proteins assessed by subjecting cells to immunofluorescence using PAR-specific antibodies. Robust nuclear ADP-ribosylation is observed in Ax2, *adprt1a*<sup>-</sup> and *adprt2*<sup>-</sup> cells in response to MMS (Fig. 3A). Strikingly, however, ADP-ribosylation following exposure to MMS is abrogated in the *adprt1a*<sup>-</sup>*adprt2*<sup>-</sup> strain. A similar abrogation of ADP-ribosylation is observed in *adprt1a*<sup>-</sup>*adprt2*<sup>-</sup> cells exposed to H<sub>2</sub>O<sub>2</sub> (data not shown). Therefore, in the absence of Adprt2, Adprt1a undertakes a critical role in signalling base damage and DNA SSBs.

Although disruption of the *adprt2* gene induces sensitivity to agents that induce SSBs, our observations that Adprt1a is required for MMS-induced ADP-ribosylation in these cells raises the possibility that alternate pathways exist that allow cells to tolerate genotoxic stress in the absence of Adprt2. To test this possibility, we assessed the impact of disrupting *adprt1a* and *adprt2* in combination on the ability of cells to tolerate exposure to increasing concentrations of MMS. As reported previously (Couto et al., 2011), *adprt1a*<sup>-</sup> cells tolerate exposure to MMS equivalent to the parental Ax2 strain, while *adprt2*<sup>-</sup> cells are sensitive to this agent. Strikingly, however, the *adprt1a*<sup>-</sup>*adprt2*<sup>-</sup> strain exhibits increased sensitivity to MMS above that of the



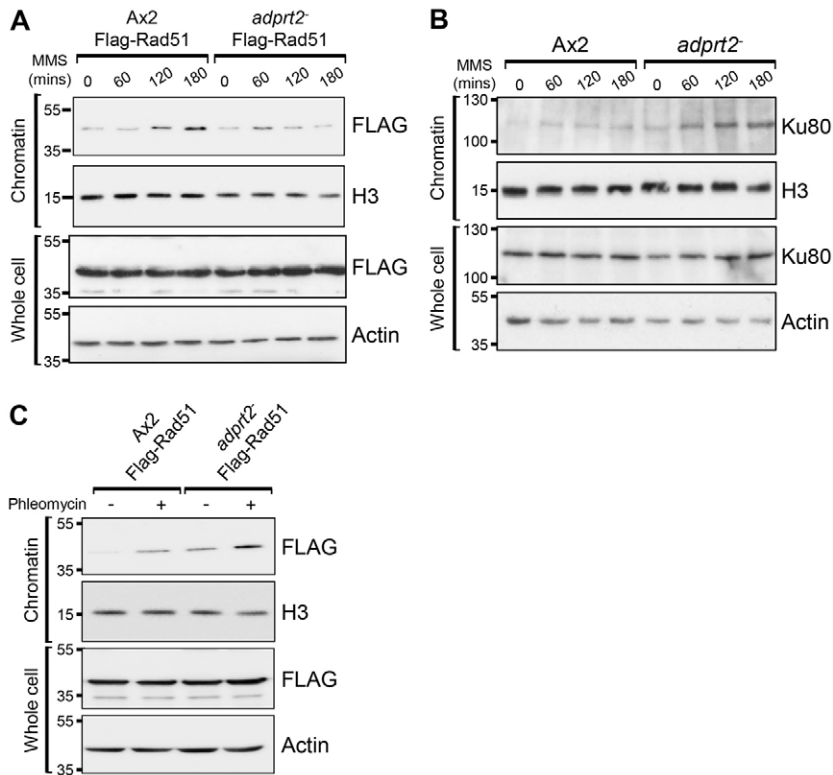
**Fig. 3. In the absence of Adprt2, Adprt1a signals MMS-induced DNA damage to maintain cell viability.** (A) Ax2, *adprt1a*<sup>-</sup>, *adprt2*<sup>-</sup> and *adprt1a*<sup>-</sup>*adprt2*<sup>-</sup> cells were left untreated (-) or exposed to 5 mM MMS for 30 minutes (+), as indicated. Cells were subjected to immunofluorescence using antibodies that recognise PAR. The percentage of PAR-positive cells was scored from a population of >200 cells. Error bars indicate the s.e.m. from three independent experiments. (B) Exponentially growing Ax2, *adprt1a*<sup>-</sup>, *adprt2*<sup>-</sup> and *adprt1a*<sup>-</sup>*adprt2*<sup>-</sup> cells were assessed for their ability to survive exposure to increasing concentrations of MMS. Error bars indicate the s.e.m. from three independent experiments.

single mutants (Fig. 3B). A similar increased sensitivity of the *adprt1a*<sup>-</sup>*adprt2*<sup>-</sup> strain was observed in response to H<sub>2</sub>O<sub>2</sub> (data not shown). Taken together, these data illustrate that in the absence of Adprt2, Adprt1a is required to ADP-ribosylate substrates and promote resistance of cells to agents that induce base damage and/or DNA SSBs.

#### Loss of Adprt2 results in increased frequency of DNA DSBs following exposure to MMS that are recognised by the NHEJ machinery

Next, we considered the type of DNA lesions induced by MMS in *adprt2*<sup>-</sup> cells. One possibility is that in the absence of Adprt2, Adprt1a undertakes a hitherto unrecognised role in signalling SSBs. However, given that Adprt1a is required to ADP-ribosylate proteins following DNA DSBs (Couto et al., 2011), we also considered the possibility that deregulated SSBs results in formation of DNA DSBs that are subsequently signalled by Adprt1a. To test this hypothesis we compared enrichment of HR and NHEJ factors at DNA lesions in *adprt2*<sup>-</sup> and parental Ax2 cells following exposure to MMS. Initially, we assessed the ability of an HR factor to recognise DNA lesions by expressing FLAG-tagged Rad51 in Ax2 and *adprt2*<sup>-</sup> cells. Compared to untreated cultures, increased levels of FLAG-Rad51 are apparent in chromatin fractions prepared from Ax2 cells treated with MMS (Fig. 4A; supplementary material Fig. S2A). In addition to inducing DNA damage during DNA replication, high doses of base damage can produce a DNA DSB when two lesions in close





**Fig. 4. In the absence of Adprt2, MMS-induced DNA damage is converted to DNA DSBs and detected by components of the NHEJ pathway.** (A) Ax2 and *adprt2*<sup>-</sup> cells expressing FLAG–Rad51 were left untreated or were exposed to 5 mM MMS. At the indicated times following addition of MMS (minutes), subcellular fractionation was performed and chromatin or whole cell extracts analysed by western blotting using the indicated antibodies. (B) Ax2 and *adprt2*<sup>-</sup> cells were left untreated or were exposed to 5 mM MMS. At the indicated times following addition of MMS (minutes), subcellular fractionation was performed and chromatin or whole cell extracts analysed by western blotting using the indicated antibodies. (C) Ax2 and *adprt2*<sup>-</sup> cells expressing FLAG-tagged Rad51 were left untreated or exposed to 200 µg/ml phleomycin for 90 minutes. Following subcellular fractionation, chromatin or whole cell extracts were analysed by western blotting using the indicated antibodies.

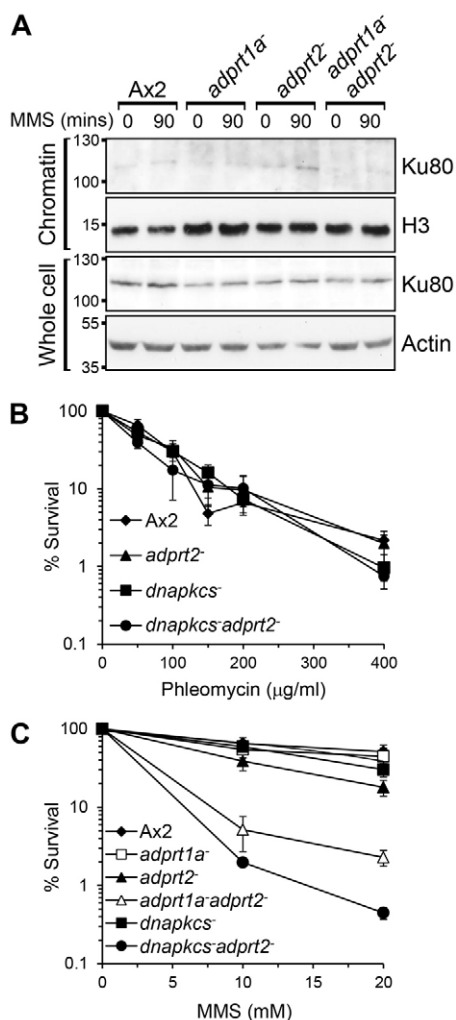
proximity are processed. This, taken together with our observations that low levels of H2AX phosphorylation are observed in Ax2 cells following exposure to MMS (Fig. 1A,B), suggest that enrichment of FLAG–Rad51 in chromatin prepared from these cells is a result of DNA DSBs induced by prolonged exposure of cells to this agent. In contrast to Ax2 cells, no significant enrichment of FLAG–Rad51 is observed in chromatin fractions following exposure of *adprt2*<sup>-</sup> cells to MMS, indicating that HR is not engaged in these cells in response to this agent (Fig. 4A; supplementary material Fig. S2A). In contrast, while no gross enrichment of Ku80 is observed in chromatin fractions prepared from Ax2 cells following treatment with MMS, enrichment of Ku80 in chromatin is observed in *adprt2*<sup>-</sup> cells (Fig. 4B; supplementary material Fig. S3A). Taken together, these data indicate that in the absence of Adprt2, a proportion of MMS-induced DNA lesions are converted to DNA DSBs that are subsequently recognised by the NHEJ machinery.

The lack of FLAG–Rad51 recruitment to MMS-induced DNA damage in *adprt2*<sup>-</sup> cells is surprising given that this factor is recruited to DNA lesions in Ax2 cells (Fig. 4A; supplementary material Fig. S2A). One interpretation of these data are that in the absence of Adprt2, MMS-induced DNA damage is specifically recognised by components of the NHEJ machinery. However, given that ARTs have been implicated promoting HR at stalled and/or collapsed replication forks (Bryant et al., 2009; Sugimura et al., 2008; Yang et al., 2004), an alternative possibility is that this observation reflects a defect in the HR pathway in *adprt2*<sup>-</sup> cells, as opposed to an inherent bias towards NHEJ. However, compared to untreated control cultures, FLAG–Rad51 is enriched in chromatin fractions prepared from Ax2 and *adprt2*<sup>-</sup> cells exposed to the DSB-inducing agent phleomycin (Fig. 4C; supplementary material Fig. S2B). Further, integration of a

hygromycin resistance cassette using targeted HR is apparent at the *cdk8* locus in  $9.4 \pm 2.9\%$  of *adprt2*<sup>-</sup> cells that have integrated the knockout construct into their genome. Taken together, these data indicate that *adprt2*<sup>-</sup> cells are able to perform HR and gene conversion events at canonical DNA DSBs. Therefore, in the absence of Adprt2, MMS-induced DNA lesions are converted to DNA DSBs with increased frequency and are specifically channelled through the NHEJ pathway as opposed to HR.

#### In the absence of Adprt2, NHEJ promotes resistance to base damage induced by MMS

Although disruption of the *adprt2* gene induces sensitivity to DNA SSBs, our observations that the *adprt1a*<sup>-</sup>*adprt2*<sup>-</sup> strain exhibits abrogated ADP-ribosylation and increased sensitivity to MMS indicates that Adprt1a regulates alternate repair pathways to confer resistance of *adprt2*<sup>-</sup> cells to this agent. Adprt1a promotes NHEJ by facilitating enrichment of Ku at DNA DSBs (Couto et al., 2011; Pears et al., 2012). Given that Ku is enriched at MMS-induced damage in *adprt2*<sup>-</sup> cells (Fig. 4B; supplementary material Fig. S3A), we hypothesised that NHEJ may contribute to resistance of these cells to MMS. A prediction of this model would be that Adprt1a is similarly required to enrich Ku at MMS-induced DNA damage in *adprt2*<sup>-</sup> cells. To test this possibility, we assessed the impact of disrupting *adprt1a* on the enrichment of Ku at MMS-induced DNA lesions in *adprt2*<sup>-</sup> cells. As expected, Ku80 is not enriched in chromatin fractions prepared from Ax2 and *adprt1a*<sup>-</sup> cells following exposure to MMS, while elevated levels of Ku80 are apparent in chromatin prepared from *adprt2*<sup>-</sup> cells following DNA damage. Interestingly, however, disruption of *adprt1a* in the *adprt2*<sup>-</sup> strain results in a decrease in Ku80 enrichment in chromatin following exposure to MMS to levels comparable with those



**Fig. 5. In the absence of Adprt2 NHEJ is required for tolerance of cells to MMS-induced DNA damage.** (A) The indicated strains were left untreated or were exposed to 5 mM MMS for 90 minutes. Following subcellular fractionation, chromatin or whole cell extracts were analysed by western blotting using the indicated antibodies. (B) Exponentially growing Ax2, *adprt2*<sup>-</sup>, *dnapkc*<sup>-</sup> and *dnapkc*<sup>-</sup>*adprt2*<sup>-</sup> cells were assessed for their ability to survive exposure to increasing concentrations of phleomycin. Error bars indicate the s.e.m. from three independent experiments. (C) The indicated mutant strains, along with parental Ax2 cells were assessed for their ability to survive exposure to increasing concentrations of MMS. Error bars indicate the s.e.m. from three independent experiments.

observed in Ax2 cells (Fig. 5A; supplementary material Fig. S3B). Therefore, in the absence of Adprt2, Adprt1a is required to facilitate assembly of NHEJ factors at MMS-induced DNA lesions.

Given that Adprt1a is required to promote resistance of *adprt2*<sup>-</sup> cells to MMS, our observations that Adprt1a promotes enrichment of Ku at DNA lesions suggests that this resistance is achieved by engaging the NHEJ pathway. To test this possibility, we generated a strain in which the *adprt2* gene is disrupted in combination with DNA-PKcs, a component of the core NHEJ pathway conserved in *Dictyostelium* (Hudson et al., 2005). Importantly, *dnapkc*<sup>-</sup>*adprt2*<sup>-</sup> cells are not sensitive to DNA DSBs above parental Ax2 cells or single mutants (Fig. 5B).

Given that disruption of HR is either lethal to cells, or induces sensitivity to DSBs in vegetative *Dictyostelium* (Hsu et al., 2011), these data indicate that similar to the *adprt2*<sup>-</sup> strain, *dnapkc*<sup>-</sup>*adprt2*<sup>-</sup> cells are competent for HR. Strikingly, however, while disruption of *dnapkc* alone does not result in sensitivity of cells to MMS, loss of this gene in combination with *adprt2* further sensitises these cells to MMS (Fig. 5C). Taken together, these data indicate that in the absence of an effective SSB repair pathway, DNA lesions are converted to DSBs that are subsequently detected and resolved by the NHEJ pathway.

## Discussion

Previously, we illustrated that Adprt1b and Adprt2 are required for tolerance of *Dictyostelium* cells to agents that induce base damage and DNA SSBs (Couto et al., 2011; Pears et al., 2012). Here we extend these studies and report that loss of Adprt2 results in genome instability leading to conversion of DNA SSBs to DSBs that are subsequently repaired by NHEJ. Compared to parental Ax2 cells, *adprt2*<sup>-</sup> cells exhibit elevated levels of H2AX phosphorylation in the absence of genotoxic stress that is further increased when cells are exposed to agents that induce base damage and/or DNA SSBs. Mechanistically, this increased DNA damage may occur through several distinct but non-mutually exclusive mechanisms. For example, DNA damage may occur as a consequence of unrepaired SSBs being converted to DSBs as replication forks encounter DNA lesions during S-phase. Alternatively, the inability of cells to repair SSBs may result in formation of DSBs either as a consequence of two unrepaired DNA lesions occurring in close proximity, or through deregulated repair leading to illegitimate processing of DNA damage. Interestingly, we observe low levels of H2AX phosphorylation in parental Ax2 cells following exposure to MMS. The number of cells that exhibit this damage is equivalent to those undergoing DNA replication at the time of exposure to DNA damage. Further, we observe a proportional increase in the number of nuclei that exhibit  $\gamma$ H2AX staining in response to MMS with those undergoing S-phase in synchronous cell cultures. This correlation is consistent with Ax2 cells accumulating additional DNA damage during DNA replication, as opposed to illegitimate processing of DNA damage during other phases of the cell cycle. In contrast, *adprt2*<sup>-</sup> cells exhibit a greater number of cells that exhibit H2AX phosphorylation than those undergoing DNA replication in the absence or presence of MMS (Fig. 2A–C). In addition, the number of  $\gamma$ H2AX-positive cells does not further increase when cell populations are enriched in S-phase prior to exposure to MMS (Fig. 2C,D). This lack of an obvious correlation between the increased H2AX phosphorylation and DNA replication in *adprt2*<sup>-</sup> cells illustrates that in contrast to Ax2 cells DNA damage is not restricted to S-phase but can occur at other stages of the cell cycle. Given that vegetative *Dictyostelium* cells have no discernible G1 (Muramoto and Chubb, 2008; Weijer et al., 1984), we believe this occurs in G2-phase of the cells cycle, or as cells undergo mitosis.

While *adprt2*<sup>-</sup> cells are sensitive to agents that induce base damage and SSBs, ADP-ribosylation remains intact in these cells (Couto et al., 2011). These data illustrate that in the absence of Adprt2, other ARTs signal DNA damage induced by MMS and H<sub>2</sub>O<sub>2</sub>. This is reminiscent of the situation in vertebrates. For example, depletion of PARP1 similarly results in increased levels of H2AX phosphorylation following exposure to H<sub>2</sub>O<sub>2</sub> (Woodhouse et al., 2008) and PARP2 has been implicated in

catalysing the reduced levels of ADP-ribosylation following strand breakage in *parp1*<sup>-/-</sup> cells (Amé et al., 1999). Unfortunately, the inability to disrupt murine *parp1* and *parp2* in combination prohibits testing this model in defined genetic backgrounds (Ménissier de Murcia et al., 2003). However, depletion of PARP2 alone or in combination with PARP1 does not dramatically impact on the ability of human cells to repair DNA strand breaks (Fisher et al., 2007), suggesting ARTs other than PARP2 are responsible for signalling DNA damage in the absence of PARP1. Unfortunately, we have similarly been unable to disrupt the two *Dictyostelium* SSB responsive ARTs (Adprt1b and Adprt2) in combination. Therefore, we have been unable to test whether these ARTs act together to signal DNA SSBs. Recently, however, we identified an additional ART required for resolution of DNA DSBs (Adprt1a), raising the possibility of further cross-talk between ARTs in the DDR (Couto et al., 2011). In this regard, we find that disrupting *adprt1a* and *adprt2* in combination abrogates ADP-ribosylation in response to MMS and H<sub>2</sub>O<sub>2</sub> and further sensitises cells to these agents. These data illustrate that in the absence of Adprt2, Adprt1a undertakes a hitherto unrecognised role of signalling DNA damage to maintain cell viability following base damage and/or SSBs. It should be noted that similar to *Dictyostelium* Adprt1a, PARP3 was recently identified as an additional ART that ADP-ribosylate proteins at DNA lesions to facilitate NHEJ (Rulten et al., 2011). It is interesting to speculate, therefore, that similar cross-talk between PARP1 and PARP3 is possible when signalling DNA damage and that this ART is responsible for the residual ADP-ribosylation observed in *parp1*<sup>-/-</sup> cells. In this regard, PARP1 and PARP3 act synergistically in response to IR in mouse and human cells (Boehler et al., 2011). In light of our data it will be of interest to more formally test the relationship between PARP1 and PARP3 in signalling DNA damage to facilitate repair following DNA SSBs and other varieties of DNA damage.

Several possibilities could explain how Adprt1a-mediated signalling of DNA damage promotes resistance of *adprt2*<sup>-</sup> cells to agents that induce base damage and DNA SSBs. For example, Adprt1a may act analogous to Adprt2 and undertake a hitherto unrecognised role in signalling DNA damage to promote canonical SSBR pathways. However, given that H2AX phosphorylation is a commonly used marker for DSB induction, we reasoned that the robust induction of H2AX phosphorylation in *adprt2*<sup>-</sup> cells may indicate formation of DNA DSBs in these cells. Our observations that Adprt1a is required to signal DNA DSBs (Couto et al., 2011), in addition to our findings that Adprt1a signals MMS-induced DNA damage in the absence of Adprt2, prompted us to assess whether SSBs are converted to DSBs that are subsequently recognised by components of the DSB repair machinery. We find that while *adprt2*<sup>-</sup> cells exhibit slightly elevated levels of Rad51 in chromatin in the absence of genotoxic stress, this is not further increased when robust induction of H2AX phosphorylation is observed in these cells following exposure to MMS. Instead, we observe enrichment of Ku at DNA lesions. Taken together these data indicate that deregulated signalling of SSBs in the absence of Adprt2 results in DSB formation that is subsequently recognised by components of the NHEJ pathway.

It is important to note that vertebrate ARTs have been implicated in promoting HR, particularly at DSBs generated at stalled replication forks (Bryant et al., 2009; Sugimura et al., 2008; Yang et al., 2004). Given that Adprt2 is acting in a manner

analogous to PARP1, at least as regards signalling base damage and DNA SSBs, an important consideration is that the absence of FLAG-Rad51 recruitment to MMS-induced DNA lesions in *adprt2*<sup>-</sup> cells reflects an inherent defect in HR, as opposed to a preferential engagement of the NHEJ machinery. However, we are able to disrupt genes by targeted HR in the *adprt2*<sup>-</sup> strain, and Rad51 is recruited to canonical DNA DSBs induced in these cells using phleomycin. Further, while disruption of HR genes in *Dictyostelium* results in either cell death (Hudson et al., 2005; Zhang et al., 2009) or sensitivity to DNA DSBs (Hsu et al., 2011), no such phenotype is observed in *adprt2*<sup>-</sup> cells (Couto et al., 2011). Therefore, we believe *adprt2*<sup>-</sup> cells are competent for HR and favour the possibility that the increased formation of DSBs observed in these cells in response to MMS are specifically channelled through the NHEJ pathway. In this regard, it is interesting to note that our work in *Dictyostelium* (Hsu et al., 2011) and that of others in vertebrates (Shibata et al., 2011) suggests that NHEJ is attempted first at DSBs and if ineffective HR is engaged. An interesting possibility, therefore, is that DSBs generated as a consequence of ineffective SSBR in *adprt2*<sup>-</sup> cells are first detected and repaired efficiently by the NHEJ machinery, rendering the HR pathway redundant or of limited importance in this context.

Taken together, our data point to alternative ARTs signalling DNA damage in the absence of canonical pathways used to repair a specific variety of DNA lesion. Specifically, we propose that in the absence of Adprt2, MMS-induced DNA lesions are converted to DSBs that are subsequently channelled through the NHEJ pathway. Adprt1a is required to enrich and/or retain Ku at DNA lesions to facilitate NHEJ (Couto et al., 2011). We similarly find that in the absence of Adprt2, Adprt1a is required for optimal enrichment of Ku at MMS-induced DNA lesions. These data, taken together with our observations that Adprt1a is required for *adprt2*<sup>-</sup> cells to tolerate SSBs, suggest that in the absence of effective SSBR, DNA lesions are converted into DNA DSBs that are subsequently repaired by NHEJ to promote cell survival. In support of this model, we find that disruption of a canonical NHEJ factor (DNA-PKcs) in the *adprt2*<sup>-</sup> strain further sensitises cells to MMS. Intriguingly, the *dnapks*<sup>-</sup>*adprt2*<sup>-</sup> strain is more sensitive to MMS than the *adprt1a*<sup>-</sup>*adprt2*<sup>-</sup> strain (Fig. 5C). In this regard, it is noteworthy that although the *adprt1a*<sup>-</sup> strain is defective in NHEJ, it does not exhibit complete abrogation of Ku enrichment at DSBs (Couto et al., 2011). Given the critical role of DNA-PKcs in NHEJ, a possible explanation for the increased sensitivity of the *adprt2*<sup>-</sup>*dnapks*<sup>-</sup> strain to MMS is that it has a more severe NHEJ defect than the *adprt1a*<sup>-</sup>*adprt2*<sup>-</sup> strain.

In contrast to our observations, disruption of the NHEJ pathway can suppress the sensitivity of Fanconi anaemia (FA) and HR-defective cells to genotoxic agents (Adamo et al., 2010; Bouwman et al., 2010; Bunting et al., 2010; Pace et al., 2010). These data indicate that under certain circumstances NHEJ is toxic to cells. It is interesting to note, however, that the instances when NHEJ is toxic are usually associated with deregulated repair of DNA DSBs during S-phase. Our data indicate that disruption of Adprt2 leads to increased formation of DSBs throughout the cell cycle. It is interesting to speculate, therefore, that while engaging the NHEJ pathway during DNA replication is undesirable, it can be potentially beneficial during other phases of the cell cycle such as G2 and compensate to some degree with the loss of an effective SSBR pathway.



In summary we show that the sensitivity of *adprt2*<sup>-</sup> cells to base damage and DNA SSBs is reflected in increased levels of DNA damage either in the absence or presence of genotoxic stress. DNA damage is not restricted to S-phase, but accumulates throughout the cell cycle and manifests itself as an increased frequency of DNA DSBs, the signalling of which becomes reliant on Adprt1a. Our findings additionally illustrate that while *adprt2*<sup>-</sup> cells are competent for HR, DSBs generated in these cells following exposure to MMS are not repaired by this pathway. Instead, they are channelled into the NHEJ pathway through Adprt1a-dependent accumulation of Ku at DNA lesions. This is reflected in an increased reliance on NHEJ to tolerate base damage in the absence of effective signalling of DNA damage by Adprt2. Taken together, these data illustrate cross-talk between different ARTs to signal DNA damage and promote alternate DNA repair pathways to maintain cell viability following genotoxic stress.

## Materials and Methods

### Cell culture and strain generation

*Dictyostelium* strains were grown using standard procedures, either axenically or on SM agar in association with *Klebsiella aerogenes*. The *dnapks*<sup>-</sup>, *adprt1a*<sup>-</sup>, *adprt2*<sup>-</sup> and *adprt1a*<sup>-</sup>*adprt2*<sup>-</sup> strains have been previously described (Couto et al., 2011; Hudson et al., 2005). To generate the *dnapks*<sup>-</sup>*adprt2*<sup>-</sup> strain, the *adprt2* disruption construct (Couto et al., 2011) was transfected into the *dnapks*<sup>-</sup> strain in which the BSR cassette had been removed using cre-recombinase (Hudson et al., 2005). Following selection, blasticidin-resistant clones were isolated using standard procedures, and gene disruption verified by PCR and Southern blot (supplementary material Fig. S1). All key observations were repeated in more than one independent clone.

The cell synchronisation protocol was adapted from previously described methods (Araki et al., 1994; Strasser et al., 2012). Briefly, exponentially growing cells were seeded at between 0.75 and 1×10<sup>6</sup> cells/ml and incubated in shaking suspension (220 rpm) at 9.5°C for 16 hours to induce cell cycle arrest in G2. Subsequently, cells were released from this block by raising the temperature of cultures to 22°C within 30 seconds and incubating cells in shaking suspension at 22°C. Synchronicity of cell cycle progression following temperature shift to 22°C was assessed by two criteria. The cell density of synchronised cells, alongside exponentially growing cells diluted to 0.75×10<sup>6</sup>–1×10<sup>6</sup> cells/ml, was measured every hour using a haemocytometer to assess when cells divided. S-phase in synchronised cultures was predicted to occur following synchronous cell doubling ~3–4 hours post-temperature shift. The timing of S-phase in synchronised cultures was also established by scoring cells for incorporation of BrdU into DNA in each experiment (see below).

### Detecting S-phase cells by incorporation of BrdU into DNA

Asynchronous or synchronised cell cultures were allowed to settle on coverslips for 10 minutes before removing the media and replacing it with HL5 supplemented with 100 μM BrdU either in the presence or absence of MMS. After 30 minutes coverslips were incubated for 5 minutes in ice-cold nuclear extraction buffer (10 mM Pipes, pH 6.8, 300 mM sucrose, 3 mM MgCl<sub>2</sub>, 20 mM NaCl and 0.5% Triton X-100) and washed in TBS before being fixed with 70% ethanol for a further 5 minutes. Coverslips were finally rinsed in 100% methanol and washed twice with TBS. Coverslips were then incubated for 1 hour at room temperature with 2N HCl to denature DNA before being washed a further three times in TBS. Following blocking in 10% donkey serum diluted in TBS (D-TBS) for 1 hour at room temperature, coverslips were incubated overnight at 4°C with the BrdU antibody diluted in D-TBS. The following day, coverslips were washed three times in TBS and incubated with a Cy3 conjugated secondary antibody (Jackson Laboratories) diluted in D-TBS. Following three washes in TBS, coverslips were mounted onto glass slides with VECTASHIELD mounting medium containing DAPI (Vector Laboratories) and visualized as described in protocols for immunofluorescence.

### Immunoblotting and antibodies

Whole-cell extracts were prepared as described previously (Couto et al., 2011). Antibodies were obtained from the following sources: Ku80 (Hudson et al., 2005), γH2AX (Abcam), H3 (Abcam), α-Actin (Santa Cruz Biotechnology, Inc.), FLAG (Sigma-Aldrich), Myc (Cell Signalling Technology) and BrdU (Roche).

### Immunofluorescence

Immunofluorescence was carried out as described previously (Couto et al., 2011). Briefly, cells were allowed to adhere to coverslips for 1 hour before incubation in

media containing MMS at the doses and times stated in the figure legend. Coverslips were incubated for 5 minutes in ice-cold nuclear extraction buffer [10 mM PIPES (pH 6.8), 300 mM sucrose, 3 mM MgCl<sub>2</sub>, 20 mM NaCl and 0.5% Triton X-100] and washed in TBS before being fixed with 70% ethanol for a further 5 minutes. Coverslips were finally rinsed in 100% methanol and washed twice with TBS.

For detecting PAR and γH2AX nuclear staining, coverslips were blocked for 1 hour at room temperature in 10% swine serum diluted in TBS (S-TBS) before incubation in the relevant antibody diluted in S-TBS for 1 hour at room temperature. Coverslips were washed three times in TBS, incubated at room temperature for 1 hour with TRITC-conjugated secondary antibody (Dako) diluted in S-TBS, and washed three times more with TBS. Coverslips were mounted onto glass slides with VECTASHIELD mounting media containing DAPI (Vector Laboratories) and visualized with a microscope (IX71; Olympus) at a magnification of 100× with immersion oil (Lenzol). All microscopy was performed at room temperature. All images were acquired on a C10600-10B-H camera (Hamamatsu Photonics) using HCImage Acquisition (Hamamatsu Photonics) image software and processed in Adobe Photoshop.

### Gene cloning and expression vectors

The entire coding sequence of the *rad51-2* gene (DDB\_G0273611) was amplified from cDNA using a primer that incorporates a *KpnI* site followed by a FLAG tag onto the initiating methionine codon of the gene (5'-ATGGATGGTACCGA-TTATAAAGATGATGATGATAAAAATGGCATCAAGACAAAGACAAG-3') in conjunction with a primer that incorporates a *BamHI* site directly adjacent to the stop codon (5'-GGATCCTTATTGTTCTTTATAATCGGAGATACC-3').

The PCR product was purified and cloned into an intermediate vector (pJET; Thermo Scientific) by blunt-end ligation. The FLAG-Rad51 coding sequence was subsequently excised from pJET using *KpnI* and *BamHI* and cloned into the *KpnI* and *BamHI* sites present in the multiple cloning site of pDXA-3C.

### Subcellular fractionation

Subcellular fractionation was performed as described previously (Couto et al., 2011). Briefly, exponentially growing cells were seeded at 5×10<sup>6</sup> cells/ml and mock-treated or incubated with MMS or phleomycin at the concentrations and times indicated. Following treatment, cells were washed in ice-cold KK2 buffer and resuspended in nuclear lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 μM microcystin, 1 mM NaF, 2 mM sodium orthovanadate and protease inhibitor cocktail) containing 0.1% Triton X-100 to a final density of 3×10<sup>7</sup> cells/ml. Cells were incubated for 15 minutes at 4°C and then centrifuged at 14,000 g for 3 minutes, giving rise to supernatant and a pellet. The pellet was resuspended in nuclear lysis buffer + 0.1% Triton X-100, and incubated for an additional 15 minutes at 4°C before centrifugation at 14,000 g for 3 minutes. The resulting pellet was resuspended in nuclear lysis buffer supplemented with 200 μg/ml RNase A (Sigma-Aldrich) and incubated with rotation at room temperature for 30 minutes. Following this step, samples were centrifuged as before and the pellet resuspended in SDS loading buffer containing 100 mM DTT. Fractions were analysed by western blotting using the antibodies indicated in the figure.

### DNA damage sensitivity assays

Sensitivity assays were performed as described previously (Couto et al., 2011). In brief, exponentially growing *Dictyostelium* were seeded at a density of 1×10<sup>6</sup> cells/ml and incubated with the indicated concentrations of phleomycin (Sigma-Aldrich) or MMS (Sigma-Aldrich) at 100 rpm at 22°C. After 1 hour, cells were diluted to 1×10<sup>4</sup> cells/ml in KK2 and 250 cells plated in duplicate onto two 140-mm Petri dishes containing a lawn of *K. aerogenes* in association with SM agar. Petri dishes were incubated in the dark at 22°C, the number of colonies visible 3, 4 and 5 days after plating was scored to measure survival.

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### Author contributions

All data were generated by C.A.-M.C. with the exception of Fig. 1B (R.T.) and supplementary material Fig. S1E (D.-W.H.). A.R. helped C.A.-M.C. with assays to assess HR efficiency. S.L. generated the FLAG-Rad51 expression construct. C.J.P. and N.D.L. supervised this work. N.D.L. wrote the manuscript with contributions from C.P. and C.A.-M.C.

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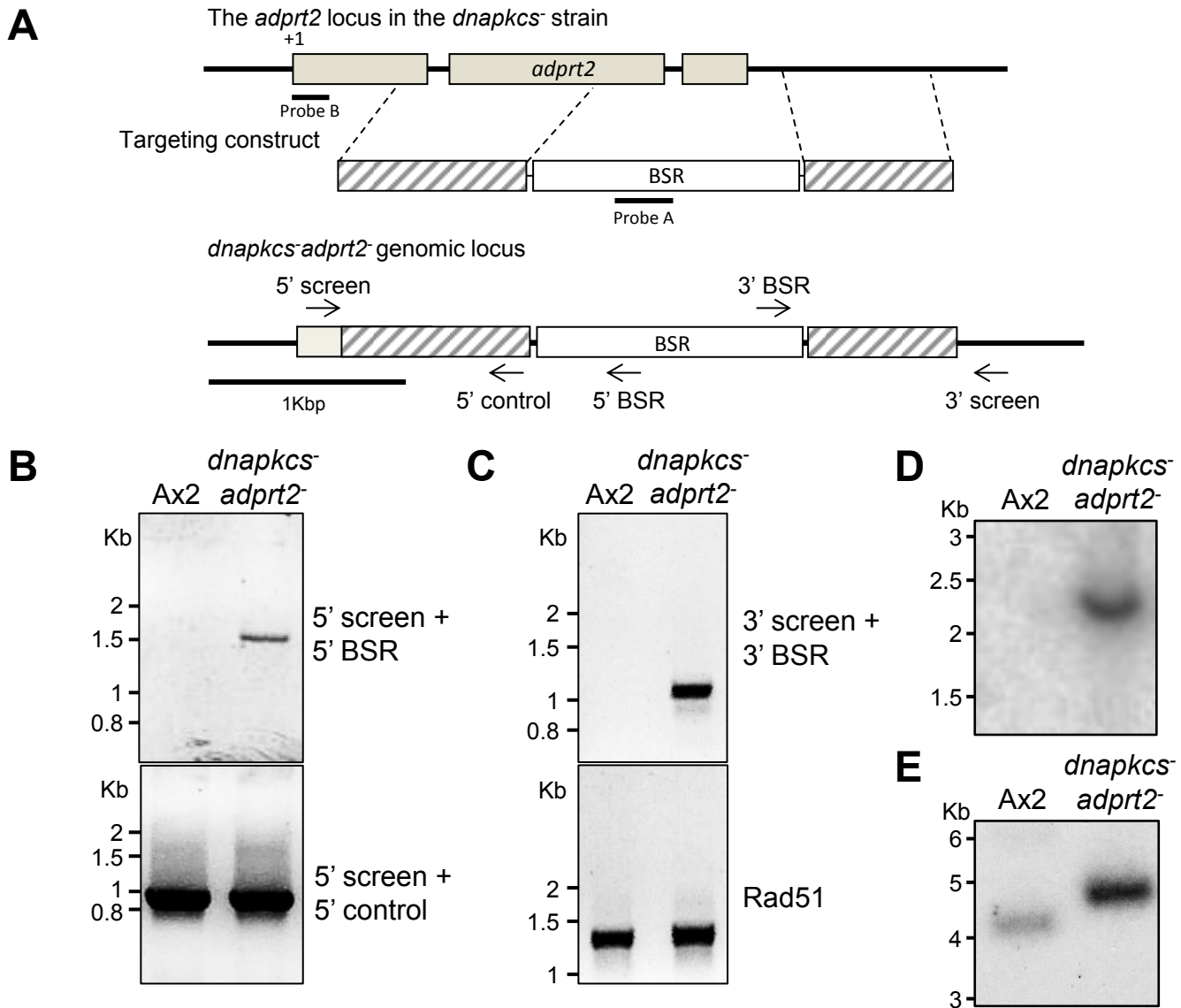
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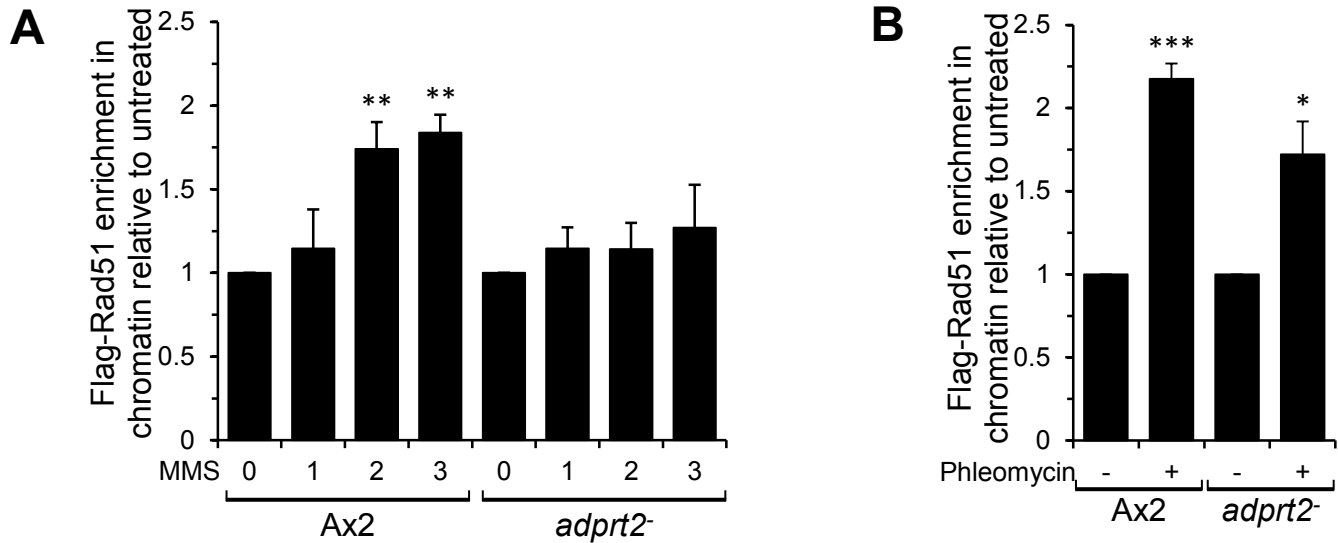
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### Supplemental Figure 1: Verification of the *dnapkcs<sup>-</sup>adprt2<sup>-</sup>* strain

**A.** Map of the *adprt2* locus in the parental *dnapkcs<sup>-</sup>* strain (upper panel) and *dnapkcs<sup>-</sup>adprt2<sup>-</sup>* disruption strain is illustrated. Hatched boxes represent regions of homology used in the disruption construct. The disruption strategy leads to replacement of the *adprt2* locus region that encodes the catalytic domain with the BSR cassette. Primers used in PCR screening for targeted integration of the BSR cassette are indicated, as are the locations of probes used in Southern blotting. **B.** The *dnapkcs<sup>-</sup>* strain was transfected with the *adprt2* disruption construct to generate the *dnapkcs<sup>-</sup>adprt2<sup>-</sup>* strain. Verification by PCR used the indicated primers. The 5' screen/5' BSR primers give rise to a 1631 base pair product only when targeted integration of the BSR cassette has occurred. The 5' screen/5' control primers are able to amplify a 958 base pair product in both Ax2 and the disruption strain. **C.** The *dnapkcs<sup>-</sup>adprt2<sup>-</sup>* strain was verified for targeted integration at the 3' end of the *adprt2* gene. The 3' screen/3' BSR primers give rise to a 1366 base pair product only when targeted integration of the BSR cassette has occurred. The Rad51 primers are able to amplify a 1234 base pairs product in both Ax2 and the disruption strain. **D.** Southern hybridisation using a probe against the BSR cassette (Probe A) on *PvuII* digested genomic DNA from Ax2 or the *dnapkcs<sup>-</sup>adprt2<sup>-</sup>* strain. Targeted integration of the BSR cassette gives rise to a predicted 2.3kb band only in the disruptant strain. **E.** Southern hybridisation using a probe against the first 189 base pairs of *adprt2* coding sequence (Probe B) on *EcoRI* digested genomic DNA from Ax2 or the *dnapkcs<sup>-</sup>adprt2<sup>-</sup>* strain. A predicted 4.1kb band is evident in Ax2 cells. Disruption of the *adprt2* gene gives rise to a predicted band of 4.5kb.

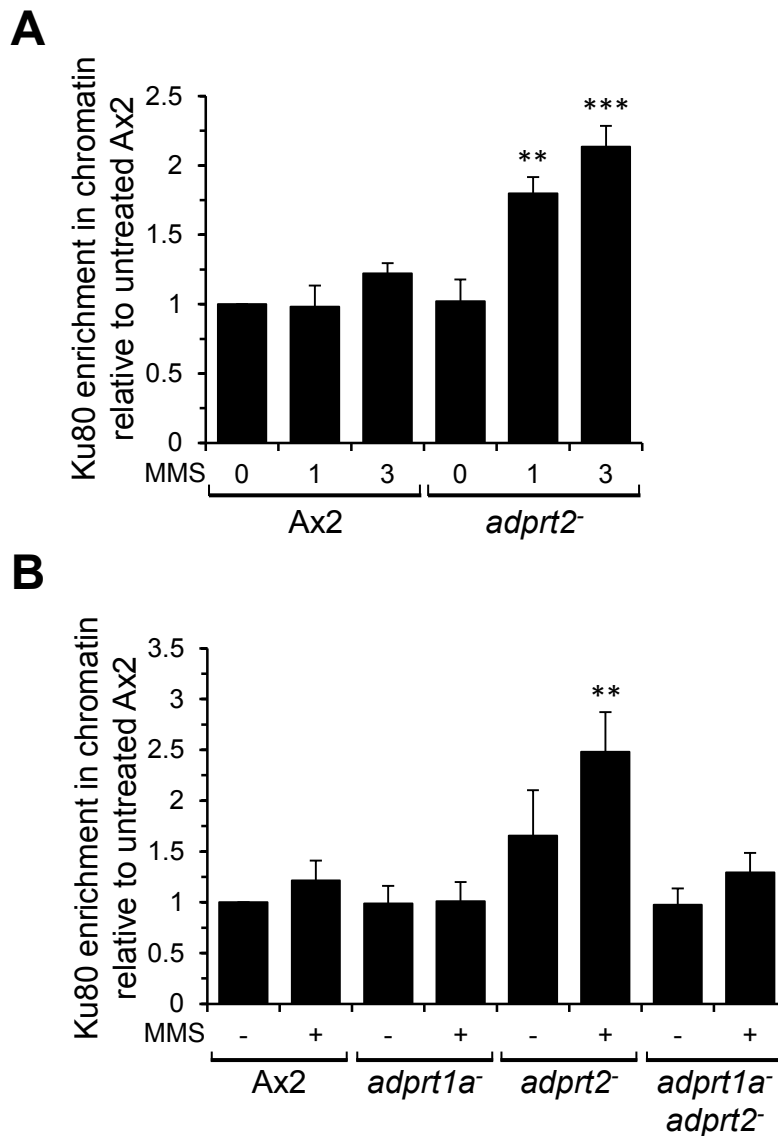




**Supplemental Figure 2:**

**A.** Quantification of Flag-Rad51 enrichment in chromatin following exposure of Ax2 and adprt2<sup>-</sup> cells to MMS. Ax2 and adprt2<sup>-</sup> cells expressing Flag-Rad51 were left untreated or exposed to 5mM MMS for indicated times (hours), chromatin fractions prepared, and western blotting performed using Flag or histone H3 antibodies. The membrane was developed with ECL and the signal quantified using the Odyssey Fc system (LI-COR). The Flag signal is normalised to the H3 signal. Data is expressed as the fold-induction of Flag-Rad51 relative to the untreated control for each strain. Error bars represent the standard error of the mean (SEM) from 3 independent experiments. \*\*, p<0.01 compared with the untreated control for each strain

**B.** Quantification of Flag-Rad51 enrichment in chromatin following exposure of Ax2 and adprt2<sup>-</sup> cells to phleomycin. Ax2 and adprt2<sup>-</sup> cells expressing Flag-Rad51 were treated with 200µg/ml phleomycin and after 90 minutes chromatin fractions prepared and analysed by Western blotting using Flag or histone H3 antibodies. The signal was detected, quantified and analysed as described in (A). Error bars represent the SEM from 3 independent experiments. \*, p<0.05; \*\*\*, p<0.001 compared with the untreated control for each strain



**Supplemental Figure 3:**

**A.** *Quantification of Ku80 enrichment in chromatin following exposure of Ax2 and adprt2<sup>-</sup> cells to MMS.* Quantification of Ku80 enrichment in chromatin after administration of MMS to Ax2 and adprt2<sup>-</sup> cells. Ax2 and adprt2<sup>-</sup> cells were left untreated or exposed to 5mM MMS for the indicated times (hours). Western blotting was performed using Ku80 or histone H3 antibodies. The membrane was developed with ECL and the signal quantified using the Odyssey Fc system (LI-COR). The Ku80 signal is normalised to the H3 signal. Data is expressed as the fold-induction of Ku80 relative to the untreated Ax2 strain. Error bars represent the standard error of the mean (SEM) from 4 independent experiments. \*\*, p<0.01; \*\*\*, p<0.001 relative to untreated Ax2.

**B.** *Quantification of Ku80 enrichment in chromatin following exposure of the Ax2, adprt1a<sup>-</sup>, adprt2<sup>-</sup> and adprt1a<sup>-</sup>adprt2<sup>-</sup> strains to MMS.* Ax2, adprt1a<sup>-</sup>, adprt2<sup>-</sup> and adprt1a<sup>-</sup>adprt2<sup>-</sup> cells were left untreated or exposed to 5mM MMS and after 90 minutes chromatin fractions prepared and analysed by Western blotting using Ku80 or histone H3 antibodies. The signal was detected, quantified and analysed as described in (A). Error bars represent the SEM from 5 independent experiments. \*\*, p<0.01 relative to the Ax2 untreated control.