



Published in final edited form as:

*J Cell Sci.* 2006 January 1; 119(Pt 1): 124–131.

## Production of reactive oxygen species in response to replication stress and inappropriate mitosis in fission yeast

Maria A Marchetti<sup>1,\*</sup>, Martin Weinberger<sup>2</sup>, Yota Murakami<sup>3</sup>, William C Burhans<sup>2,‡</sup>, and Joel A Huberman<sup>1,‡</sup>

<sup>1</sup>Department of Cancer Genetics and

<sup>2</sup>Department of Cell Stress Biology, Roswell Park Cancer Institute, Elm & Carlton Streets, Buffalo, NY 14263, USA

<sup>3</sup>Department of Viral Oncology, Institute for Virus Research, Kyoto University, Shogoinkawaharomachi, Sakyo-ku, Kyoto 606-8507, Japan

### Summary

Previous studies have indicated that replication stress can trigger apoptosis-like cell death, accompanied (where tested) by production of reactive oxygen species (ROS), in mammalian cells and budding yeast (*Saccharomyces cerevisiae*). In mammalian cells, inappropriate entry into mitosis also leads to cell death. Here we report similar responses in fission yeast (*Schizosaccharomyces pombe*). We used ROS- and death-specific fluorescent stains to measure the effects of mutations in replication initiation and checkpoint genes in fission yeast on the frequencies of ROS production and cell death. We found that certain mutant alleles of each of the four tested replication initiation genes caused elevated ROS and cell death. Where tested, these effects were not enhanced by checkpoint gene mutations. Instead, when cells that were competent for replication but defective in both the replication and damage checkpoints were treated with hydroxyurea, which slows replication fork movement, the frequencies of ROS production and cell death were greatly increased. This was a consequence of elevated CDK activity, which permitted inappropriate entry into mitosis. Thus studies in fission yeast are likely to prove helpful in understanding the pathways that lead both from replication stress and from inappropriate mitosis to cell death in mammalian cells.

### Keywords

reactive oxygen species; cell death; checkpoint; replication; mitosis; apoptosis

### Introduction

The term “reactive oxygen species (ROS)” applies to any mixture of molecules, ions and free radicals containing derivatives of molecular oxygen that are more reactive than oxygen itself. The ROS formed in living cells commonly include hydrogen peroxide, hydroxyl radical and superoxide anion. The normal process of respiration in mitochondria is a major source of ROS, and production of ROS is enhanced when mitochondrial function is disturbed during apoptosis. In apoptosis, and also in some types of necrotic cell death, unusually large amounts of ROS can be released and can contribute, by extensive oxidation of macromolecules, to the killing of cells. In some types of apoptosis, ROS also serve essential signaling functions (reviewed in (Fleury et al., 2002)).

<sup>‡</sup> Authors for correspondence (e-mail: wburhans@acsu.buffalo.edu, huberman@buffalo.edu).

\*Present address: Florida Atlantic University, Charles E. Schmidt College of Science, 777 Glades Road, P.O. Box 3091, Boca Raton, FL 33431

Apoptosis is not confined to multicellular eukaryotes. Unicellular organisms, including budding yeast and fission yeast, can undergo programmed cell death with many of the features of apoptosis in multicellular organisms (reviewed in (Burhans et al., 2003; Madeo et al., 2004; Rodriguez-Menocal and D'Urso, 2004)). Where tested, ROS production has proved to accompany apoptosis in yeasts and fungi (Balzan et al., 2004; Cheng et al., 2003; Madeo et al., 1999; Zhang et al., 2003), although in some cases it is not required (Balzan et al., 2004; Cheng et al., 2003).

A wide variety of factors can stimulate apoptosis in yeasts (reviewed in (Burhans et al., 2003; Madeo et al., 2004)). In budding yeast these apoptotic triggers include extensive DNA damage (Blanchard et al., 2002; Qi et al., 2003; Weinberger et al., 2005) and defects in the replication initiation proteins Cdc6p (Blanchard et al., 2002) and Orc2p (Weinberger et al., 2005). The same triggers, DNA damage (Norbury and Zhivotovsky, 2004) and defects in replication initiation proteins (Blanchard et al., 2002; Dodson et al., 2004; Feng et al., 2003; Kim et al., 2003; Pelizon et al., 2002; Schories et al., 2004; Shreeram et al., 2002; Yim et al., 2003), can also induce apoptosis in mammalian cells.

In mammalian cells, defects in DNA replication or DNA structure activate replication and damage checkpoints, and, among other consequences, these checkpoints inhibit CDK activity—thus preventing cells from entering mitosis with incompletely replicated or damaged DNA (Sancar et al., 2004). Entry into mitosis with damaged or incompletely replicated DNA usually leads to cell death by an apoptotic mechanism (Castedo et al., 2004). For these reasons it seemed likely to us that mutations in genes important for the DNA damage or replication checkpoints might enhance susceptibility to inappropriate mitosis when combined with mutations in genes affecting replication initiation.

Study of the interaction between checkpoint genes and replication initiation genes in pathways leading to apoptosis would be facilitated by the availability of a model organism capable of undergoing apoptosis and with checkpoint pathways similar to those of mammalian cells but more amenable to genetic analysis than mammalian cells. Fission yeast is such an organism. In contrast to budding yeast, in which checkpoints prevent mitosis primarily by inhibiting spindle elongation or anaphase chromosome separation, checkpoints in fission yeast—as in mammalian cells—prevent entry into mitosis by inhibiting CDK activity (Caspari and Carr, 1999). Furthermore, there is substantial evidence for apoptosis in fission yeast (Brezniceanu et al., 2003; James et al., 1997; Jürgensmeier et al., 1997; Zhang et al., 2003).

Here we report the use of ROS production and cell death assays in fission yeast to test our prediction that checkpoint gene mutations would enhance the effects of replication initiation gene mutations on the frequency of apoptosis-like cell death. We found that mutations in genes encoding replication initiation proteins were likely to stimulate ROS production. Where tested, this ROS production was not further stimulated by loss of checkpoint functions—in contrast to our prediction. However, we found that when replication forks were slowed by treatment with HU, checkpoint mutations dramatically stimulated ROS production and cell death. It is known that checkpoint failure leads to uncontrolled CDK activity and entry into mitosis with unreplicated DNA (Boddy et al., 1998). Cells with constitutively activated CDK also enter mitosis prematurely (Gould and Nurse, 1989), and we found that they produced ROS. These results suggest that several different pathways, with varying degrees of dependence on checkpoints, can lead from replication defects to ROS production and cell death in fission yeast.

## Materials and Methods

### Cell culture

The strains used in this study are listed in Table S1. Cells were propagated at the indicated temperatures in YES medium (Moreno et al., 1991), with the exception of the *dfp1* mutants, which were maintained in Edinburgh Minimal Medium (EMM) (Moreno et al., 1991) containing supplements with the exception of leucine.

### ROS and PI-staining assays

Logarithmically growing non-temperature-sensitive strains were incubated at 25°C. Temperature-sensitive strains in log phase were shifted from the permissive temperature (25°C) to 25°, 30°, 35° or 37° for 2, 4 or 6 hours. In indicated cases, HU (USB Corporation; 12 mM) or MG132 (Calbiochem; 250 µM) was added at the beginning of the incubation. At the end of the incubation the ROS indicator dye (DCDHFDA; Molecular Probes) was added (10 µg/ml final), and incubation was continued for 80 minutes. Cells were then harvested in a table-top centrifuge and washed twice with citrate buffer (50 mM sodium citrate, pH 7.0). The pellets were resuspended in an appropriate volume of citrate buffer containing PI (Sigma; 10 µg/ml final) and then analyzed by fluorescence microscopy or flow cytometry. The flow data were further analyzed using FlowJo software (TreeStar, Inc.) as described in Fig. S1.

### Generation of double-mutant strains

Double-mutant strains were generated by appropriate crosses followed by selection for indicator phenotypes.

### Cloning of the *orp5*<sup>+</sup> genomic fragment and cDNA and disruption of *orp5*<sup>+</sup>

The *orp5*<sup>+</sup> gene was localized to cosmid 855 (Mizukami et al., 1993), which maps just proximal to the *moc1*<sup>+</sup>/*sds23*<sup>+</sup> gene on chromosome II. The *orp5*<sup>+</sup> genomic clone was obtained by PCR and confirmed by sequencing. The full length *orp5*<sup>+</sup> cDNA was isolated from a ZapII (Stratagene) *S. pombe* cDNA library by plaque hybridization with the *orp5*<sup>+</sup> genomic fragment. Sequencing was carried out on both strands.

### Isolation of *orp5* temperature-sensitive mutants

A 4.1-kb fragment containing the *sds23*<sup>+</sup> and *orp5*<sup>+</sup> genes was amplified by PCR and cloned into pBluescript, and a 2.2-kb fragment containing *ura4*<sup>+</sup> was inserted between the *sds23*<sup>+</sup> and *orp5*<sup>+</sup> open reading frames. The resulting plasmid was used as a template for mutagenic PCR with excess dNTPs to amplify the *sds23*<sup>+</sup>, *ura4*<sup>+</sup>, *orp5*<sup>+</sup> region. The resulting PCR products were transformed into the fission yeast strain, YM71 (*h*-, *ura4D-18*, *leu1-32*, *ade6-704*). The *ura4*<sup>+</sup> transformants obtained at 25°C, and in which the chromosomal *orp5*<sup>+</sup>-*sds23*<sup>+</sup> region was replaced with a mutated PCR fragment by homologous recombination, were screened for temperature-sensitivity (Ts) at 36.5°C. The resulting Ts mutants were transformed with plasmids expressing either *orp5*<sup>+</sup> or *sds23*<sup>+</sup> to identify *orp5* Ts mutation(s). Nine Ts strains were complemented by the *orp5*<sup>+</sup> plasmid but not by the *sds23*<sup>+</sup> plasmid. The *orp5-H19*, *orp5-H30*, and *orp5-H37* mutants were chosen for further analysis.

## Results

### Measuring the production of ROS in fission yeast

We used the dye, 2',7'-dichlorodihydrofluorescein diacetate (DCDHFDA), which produces green fluorescence in the presence of ROS (LeBel et al., 1992; Tsuchiya et al., 1994), to measure ROS production in fission yeast cells. This dye is able to penetrate the membranes of living cells (LeBel et al., 1992). Consequently, it can detect ROS in both living cells and in

dead and dying cells that lack intact membranes. To distinguish living cells from dead ones, we used a second indicator dye, propidium iodide (PI) (Tsuchiya et al., 1994). PI cannot pass through the intact membranes of living cells. When PI enters a dead cell, it binds to the remaining DNA within the dead cell and fluoresces red. The fluorescence micrographs in Fig. 1 demonstrate that most wild-type fission yeast cells displayed insufficient spontaneous green or red fluorescence to be detected by a cooled charge-coupled device (CCD) camera. In contrast, cells bearing the *dfp1* $\Delta_{13-240}$  mutation (affecting the gene which encodes the fission yeast homolog of Dbf4p, the regulatory subunit of the Cdc7p kinase, which is essential for initiation of DNA replication; Table 1) displayed variable, but easily detectable, levels of both green and red fluorescence. We interpret the pure green cells as living cells (because they did not stain with PI) that were generating high ROS levels, and we interpret the pure red cells as dead or dying cells that were still sufficiently intact to retain DNA but were either not producing ROS or were unable to retain ROS due to membrane permeability. Some cells displayed both green and red fluorescence and appeared in various shades of yellow. These yellow-tinged cells constituted a significant fraction of the fluorescent cells. The presence of both green and red fluorescence in single cells is consistent with the possibility that ROS production preceded cell death and that the sequence of fluorescence was green to yellow to red. For the experiments reported here, we did not further investigate the possibility that ROS might be a cause of cell death. We simply noted a correlation between these two phenomena, as quantified in the experiments described below.

We used flow cytometry to quantitate the extents of red and green fluorescence (Fig. S1). In the following figures we report the number of cells per 10,000 examined whose green or red fluorescence exceeded a threshold that was set so as to exclude autofluorescence from unstained cells.

### **Mutants in the genes encoding the initiation proteins Orp2p, Orp5p and Cdc18p displayed increased ROS production and increased cell death**

The first step in the initiation of eukaryotic DNA replication is the association of the six proteins of the Origin Recognition Complex (ORC) with replication origins. This is followed by the additional association of Cdc6p (Cdc18p in fission yeast) and Cdt1p (Bell and Dutta, 2002). We tested the effects of mutations in the fission yeast homologs of some of these proteins on ROS production and PI staining. Fig. 2 displays a time-course and temperature-dependence study of ROS production in fission yeast strains bearing mutations in *orp2* (encoding the second largest subunit of ORC; (Kiely et al., 2000; Leatherwood et al., 1996)) or *cdc18* (encoding the fission yeast homolog of Cdc6p; (Kelly et al., 1993; Muzi-Falconi et al., 1996)). Several interesting conclusions emerge from these results. First, as in Fig. 1, there was a rough correlation between ROS production and PI staining, consistent with the possibility that ROS production is an important contributor to the cell death detected by PI staining. Second, the two mutant alleles of the *orp2* gene had strikingly different effects on ROS production and PI staining. While enhancement of ROS production in the *orp2-2* mutant strain was barely detectable even at high temperatures, enhancement in the *orp2-7* strain was striking at all temperatures and for all incubation times. Interestingly, at a restrictive temperature (36.5°C), the *orp2-2* mutation inhibits initiation of DNA replication more strongly than does the *orp2-7* mutation (Kiely et al., 2000). Furthermore, all of these mutations are temperature-sensitive for replication. Cells bearing these mutations can replicate their DNA at 25°C but not at 37°C. Therefore at lower temperatures, at least, the strong effect of the *orp2-7* mutation on ROS production and PI staining could not be a direct consequence of its inhibition of initiation of DNA replication.

In contrast to the temperature-independent effect of *orp2-7* on ROS production and PI staining, the *cdc18-K46* mutation had a temperature-dependent effect. It is possible, therefore, that ROS

production and PI staining in the *cdc18-K46* strain are direct consequences of loss of the ability of Cdc18p to contribute to formation of pre-RCs.

Similar allele-specific effects were detected when we compared mutant alleles of the *orp5* gene (which encodes the fifth largest subunit of ORC). ROS production in the *orp5-H19* strain was strong only at high temperatures, while ROS production in the *orp5-H30* strain was relatively weak at all tested temperatures (Fig. 3). Another allele, *orp5-H37*, behaved like *orp5-H30* (results not shown). As above, there was an imperfect correlation between ROS production and PI staining. However, the tested alleles of *orp5* behaved differently from those of *orp2* with regard to correlation between ROS production and replication competence. The *orp5-H19* allele showed the strongest replication-initiation defect: at 36°C, *orp5-H19* cells arrested at the G1-S interface with 1C DNA content. In contrast, *orp5-H30* cells arrested in S phase, and *orp5-H37* cells showed no obvious replication defect but arrested in G2/M with a possible mitotic defect (results not shown; F. Matsunaga, H. Kato, D. Gong, G. D'Urso, K. Tanaka and Y. Murakami, manuscript in preparation). Thus the *orp5* allele with the strongest replication defect generated the most ROS, while the *orp2* allele with the strongest replication defect generated the least ROS. This variability is consistent with other results presented in this report, which suggest that there are multiple independent pathways by which defects in replication initiation proteins can stimulate ROS production.

### N- and C-terminal deletions in the *dfp1* gene led to increased ROS production and PI staining

Dfp1p is the fission yeast homolog of budding yeast Dbf4p. Just as Dbf4p activates the Cdc7p kinase, Dfp1p activates the Hsk1p kinase, which is the fission yeast homolog of Cdc7p. In all tested eukaryotic organisms, the homologues of Cdc7p and Dbf4p are essential for initiation of DNA replication at individual replication origins (Bell and Dutta, 2002). In addition, where tested, these proteins have proved to be important for viability and checkpoint activation in response to DNA damage (reviewed in (Duncker and Brown, 2003; Kim et al., 2003)). Proteins of the Dbf4p family are not well conserved between species, except for three small motifs (N, M and C) in the N-terminal, middle and C-terminal portions of the protein, respectively (Takeda et al., 1999). Of these, motif M is essential for initiation of replication, motif N is important for protection against a wide range of DNA-damaging agents, and motif C is important specifically for protection against alkylation damage (Fung et al., 2002; Ogino et al., 2001; Takeda et al., 1999).

We wondered whether mutations in the non-essential N- and C-terminal portions of Dfp1p might lead to ROS production and cell death even in the absence of exogenous DNA-damaging agents. To test this possibility, we measured the extent of ROS production and PI staining in a series of N- and C-terminal deletion mutants that had been prepared in the laboratory of Grant Brown (Fung et al., 2002). These included three N-terminal deletions, all of which removed motif N (*dfp1*<sub>Δ183-191</sub>, *dfp1*<sub>Δ13-193</sub>, and *dfp1*<sub>Δ13-240</sub>) and two C-terminal deletions, both of which removed motif C (*dfp1*<sub>1-376</sub> and *dfp1*<sub>1-459</sub>). None of the deletions affected the essential motif M. Interestingly, we found that both N- and C-terminal deletion mutants produced ROS and stained with PI at frequencies significantly elevated compared to wild-type cells under the same conditions (Figs. 1, 4).

### Apparent replication-mutant specificity of ROS production

All of the mutations discussed above are in genes essential for initiation of DNA replication. We wanted to know whether DNA metabolism mutants that do not directly affect replication display similarly enhanced ROS production (ROS production in greater than 100 cells per 10,000). We tested the effects of deleting genes encoding proteins required for non-homologous end joining (*pku70* and *lig4* (Manolis et al., 2001)), for the DNA damage checkpoint (*rhp9* (Willson et al., 1997)), and for maintenance of replication fork stability under



stress conditions (*srs2* and *rqh1* (Maftahi et al., 2002; Marchetti et al., 2002)). We also tested genes encoding subunits of the MRN complex (*rad32* and *rad50*), which is involved in homologous recombination and checkpoint activation (Chahwan et al., 2003; Hartsuiker et al., 2001; Tavassoli et al., 1995). As shown in Fig. 5, none of the tested non-replication deletion mutants generated ROS to the same extent as certain mutant alleles of the tested genes encoding replication initiation proteins (Figs. 1–4). These results show that, in the absence of exogenous stressors, replication defects are more likely to lead to ROS production than are recombination or checkpoint defects. The differences between complete deletions of recombination and checkpoint genes (Fig. 5) and point mutations or partial deletions in replication initiation genes (Figs. 1–4) may be a simple consequence of the fact that the replication initiation proteins carry out essential functions in every cell cycle, but the functions of the tested recombination and checkpoint genes may be important only under conditions of DNA damage or replication fork block.

### Checkpoint dependence (or lack thereof) of elevated ROS production and PI staining by replication mutants

We wondered whether the increased ROS production and PI staining evident in certain replication mutant strains would be affected by inactivation of checkpoint pathways. To test this, we deleted the *rad3* gene (which encodes Rad3p, the fission yeast ATR homolog, and is essential for the damage and replication checkpoints (Bentley et al., 1996; Furuya and Carr, 2003)) or the *cds1* gene (which encodes Cds1p, the fission yeast Chk2p homolog, and is essential for the replication checkpoint (Furuya and Carr, 2003; Lindsay et al., 1998; Murakami and Okayama, 1995)) from cells bearing the *orp5-H19*, *dfp1 $\Delta$ 13–193*, or *dfp1 $\Delta$ 183–191* mutations. The results in Fig. 6 show that neither the *rad3* nor *cds1* deletion by itself had a significant effect on ROS production or PI staining. These deletions also had no significant effect on the already elevated ROS production and PI staining of the *orp5-H19* strain. In contrast, deleting either *rad3* or *cds1* inhibited the elevated ROS production and PI staining of the *dfp1* mutants. Thus a functional replication checkpoint is required for elevated ROS production by *dfp1* N-terminal deletion mutants, but ROS production in the *orp5-H19* strain is independent of both the replication and damage checkpoints. We conclude that, although many replication initiation mutants display elevated ROS production and PI staining, the pathways leading from replication defects to ROS production are not all identical. At least two pathways are involved, depending on the replication mutant. One pathway requires replication checkpoint function; the other is checkpoint-independent.

### Hydroxyurea-dependent ROS production and PI staining in checkpoint mutant strains

Although ROS production and PI staining were not significantly elevated in the *cds1* and *rad3* deletion strains (Fig. 6), we suspected that they might be elevated when these mutant strains were treated with hydroxyurea (HU), because these strains are highly sensitive to HU (Jimenez et al., 1992; Murakami and Okayama, 1995). HU inhibits ribonucleotide reductase, which leads to depletion of deoxyribonucleoside triphosphates and to the stalling of replication forks. An intact replication checkpoint involving Rad3p and Cds1p is required to maintain the stability of such stalled forks and to prevent eventual cell death (Lindsay et al., 1998; Murakami and Okayama, 1995). In the absence of the replication checkpoint, the DNA damage checkpoint (dependent on Rad3p and Chk1p) is required to inhibit rapid passage through mitosis with unreplicated DNA in the presence of HU (Boddy et al., 1998). In addition to Rad3p, both of these checkpoints require the five additional “checkpoint-Rad” proteins, Rad1p, Rad9p, Rad17p, Rad26p and Hus1p (Al-Khodairy et al., 1994). These proteins are the fission yeast homologs of the mammalian proteins with the same names, except for Rad26p, whose mammalian homolog is ATRIP (reviewed in (Nyberg et al., 2002)).

When we tested deletions of the genes encoding the checkpoint-Rad proteins we found that, in each case, treating the mutant strain with HU led to elevated ROS production and PI staining (Fig. 7). In contrast to the HU dependence of ROS production in checkpoint-Rad-mutant strains, ROS production in *dfp1* N-terminal and C-terminal deletion strains, which is high in the absence of HU, could not be significantly further stimulated by addition of HU (Fig. S2). We conclude that one or more checkpoint pathways, which are dependent on the checkpoint-Rad proteins, are required to prevent elevated ROS production and cell death when fission yeast cells are exposed to HU.

### HU-dependent, checkpoint-mutant-dependent ROS production requires inappropriate entry into mitosis

The two well-characterized checkpoint pathways operating downstream of the checkpoint-Rad proteins are the replication checkpoint, which is dependent on Cds1p (Murakami and Okayama, 1995), and the damage checkpoint, which is dependent on Chk1p (Walworth et al., 1993). To determine which of these checkpoint pathways might be required to prevent HU-induced elevated ROS production, we measured the effects on HU-induced ROS production of deleting the *cds1* and *chk1* genes. We found that neither of the single deletions facilitated ROS production or cell death during a 4-hour incubation with HU, but the HU-treated double-deletion (*cds1Δ chk1Δ*) strain produced about as much ROS and PI staining as the HU-treated *rad3Δ* strain (Fig. 8).

Since HU-treated *rad3Δ* or *cds1Δ chk1Δ* cells enter mitosis with unreplicated DNA, while HU-treated wild-type or single-mutant (*cds1Δ* or *chk1Δ*) cells do not do so after 4 hours of HU treatment (Boddy et al., 1998; Murakami and Okayama, 1995), these results suggested that the critical event triggering ROS production might be inappropriate entry into mitosis. To test this, we took advantage of the fact that inappropriate entry into mitosis can also be induced by the *cdc2-Y15F* mutation, in which tyrosine 15 of the Cdc2p cyclin-dependent kinase is replaced by phenylalanine, rendering the site non-phosphorylatable. Since phosphorylation of tyrosine 15 is a major means of negatively regulating Cdc2p kinase activity, Cdc2-Y15Fp is constitutively active. It promotes premature entry into mitosis, giving rise to shortened cells (Gould and Nurse, 1989). If inappropriate entry into mitosis is a trigger for ROS production and cell death, then *cdc2-Y15F* cells should display constitutively elevated ROS production and cell death even in the absence of HU. Indeed, that is what we found in the experiment of Fig. 8. In other cases, however, we found that—although the *cdc2-Y15F* mutation led to elevated ROS production—ROS production could be further enhanced by treatment with HU (example in Fig. S3).

## Discussion

### Defects in replication initiation proteins can induce ROS production and cell death

It is striking that at least one mutant allele of each of the tested genes encoding replication initiation proteins (*cdc18*, *dfp1*, *orp2* and *orp5*) proved capable of inducing significant ROS production and cell death (Figs. 1–4, 6). In contrast, deletions of genes encoding proteins involved in non-homologous end joining (*pku70* and *lig4*), homologous recombination (*rad32* and *rad50*), replication fork stability (*rqh1* and *srs2*), and the DNA damage checkpoint (*rhp9*) had little or no effect on ROS production (Fig. 5).

The reason for this difference between replication initiation proteins and proteins involved in other aspects of DNA metabolism is not clear. Defects in both sets of proteins would be expected to lead to increased levels of DNA damage. It is possible that the types of damage generated by replication initiation defects are distinguishable from the types of damage generated by defects in the other pathways that we studied. It is also possible that the high-

ROS-generating mutant alleles of replication initiation genes produce more spontaneous DNA damage than do deletions of the tested recombination, fork stability and checkpoint genes.

### **ROS production stimulated by defects in replication initiation proteins is not further enhanced by checkpoint mutations**

Rad3p and Cds1p are both essential for DNA-integrity checkpoints during S phase in fission yeast (Furuya and Carr, 2003; Rhind and Russell, 2000). We suspected that these replication checkpoint proteins would help to protect cells from problems generated by defects in replication initiation proteins. However, when deletions of the *rad3* or *cds1* gene were combined with mutations affecting replication initiation proteins (Orp5p or Dfp1p), no enhancement of ROS production or cell death was detected (Fig. 6). In fact, in one case (Dfp1p), deletion of *rad3* or *cds1* reduced both ROS production and cell death to near background levels, implying that ROS production and cell death in these *dfp1* mutant cells required a functional replication checkpoint. Since the *rad3* and *cds1* deletions had no effect on ROS production and cell death when combined with the *orp5-H19* mutation (Fig. 6), we conclude that defects in replication initiation proteins lead to ROS production and cell death by at least two different pathways. One pathway requires a functional replication checkpoint and the other does not. The pathway that requires a functional replication checkpoint is reminiscent of the checkpoint-dependent pathways that, in response to replication stress, induce apoptosis in mammalian cells and thus prevent cancer (reviewed in (Venkitaraman, 2005)).

### **HU-induced replication stress, combined with checkpoint mutations, leads to ROS production and cell death**

We did, however, find one mechanism by which checkpoint mutations could stimulate ROS production and cell death. When replication forks were slowed by treating cells with HU, then deletion of any of the checkpoint-Rad genes, or *chk1* plus *cds1* together, led to a striking increase of ROS production and cell death (Figs. 7, 8). The fact that significant ROS production required simultaneous deletion of *cds1* and *chk1* suggested that both the Cds1p-dependent replication checkpoint and the Chk1p-dependent damage checkpoint were individually capable of preventing ROS production. Since each checkpoint by itself is capable of down-regulating the Cdc2p cyclin-dependent kinase sufficiently to prevent premature entry into mitosis during a 4-hour incubation with HU (Boddy et al., 1998; Caspari and Carr, 1999), it seemed likely that inappropriate entry into mitosis in cells with unreplicated DNA might be the ultimate cause of the elevated ROS production and cell death induced by HU treatment of checkpoint-defective cells (Figs. 7, 8).

### **CDK dysregulation induces ROS production**

To test this possibility, we measured ROS production in a fission yeast strain carrying an altered version of the gene encoding Cdc2p (the fission yeast CDK), in which tyrosine 15 had been mutated to phenylalanine (*cdc2-Y15F*). This form of Cdc2p is constitutively active (Gould and Nurse, 1989) and cannot be down-regulated by the Cds1p- or Chk1p-dependent checkpoint pathways. We found that cells bearing this mutation frequently produced elevated ROS and died even in the absence of HU (Figs. 8, S3). These results imply that dysregulation of CDK activity and inappropriate entry into mitosis can be significant causes of ROS production and cell death in fission yeast cells, just as they are in mammalian cells (Castedo et al., 2004; Castedo et al., 2002).

### **Utility of fission yeast for study of ROS production and cell death**

Whether the ROS production and cell death that take place in certain replication initiation mutants and in HU-treated checkpoint-deficient cells are due to apoptosis or necrosis, they seem sufficiently similar to the ROS production and apoptosis that take place in mammalian



and budding yeast cells in response to initiation defects and replication stress that it is worth considering the possible utility of fission yeast as a model organism for further studies of these cell death responses. Fission yeast offers several advantages for such studies. Like budding yeast, it is a genetically tractable organism with a completely sequenced genome. In fission yeast the pathways leading to cell death include one pathway that is initiated by inappropriate mitosis and two that are initiated by defects in replication initiation proteins (Fig. 9). The first pathway is strikingly enhanced by simultaneous defects in the replication and damage checkpoints, while the second and third pathways are checkpoint-independent and dependent on a functional replication checkpoint, respectively. This multiplicity of cell death pathways in fission yeast reminds one of the multiplicity of cell death pathways in mammalian cells. Further examination of the mechanisms of these pathways in fission yeast is likely to shed light on the mechanisms by which inappropriate mitosis and defects in replication initiation proteins lead to cell death in mammalian cells.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

### Acknowledgements

Hiroaki Kato and Katsunori Tanaka contributed to the identification, characterization and mutagenesis of *orp5*. Joseph Goldbeck assisted with the initial ROS experiments. Andrew Phillips and members of the Huberman and Burhans laboratories provided useful comments on the manuscript. Grant Brown, Tony Carr, Kathy Gould, Tom Kelly, Janet Leatherwood, and Hiroto Okayama provided strains. Research in the Murakami laboratory was supported by a Grant-in-Aid for Scientific Research (B) and a Grant-in-Aid for Scientific Research on Priority Areas (A). Research in the Burhans and Huberman laboratories was supported by NIH grants CA084086 (WCB), CA095908 (JAH) and GM070566 (JAH), as well as by the Cancer Center Support Grant (P30 CA016056) to Roswell Park Cancer Institute, which partially supports the Flow Cytometry Facility employed in this study.

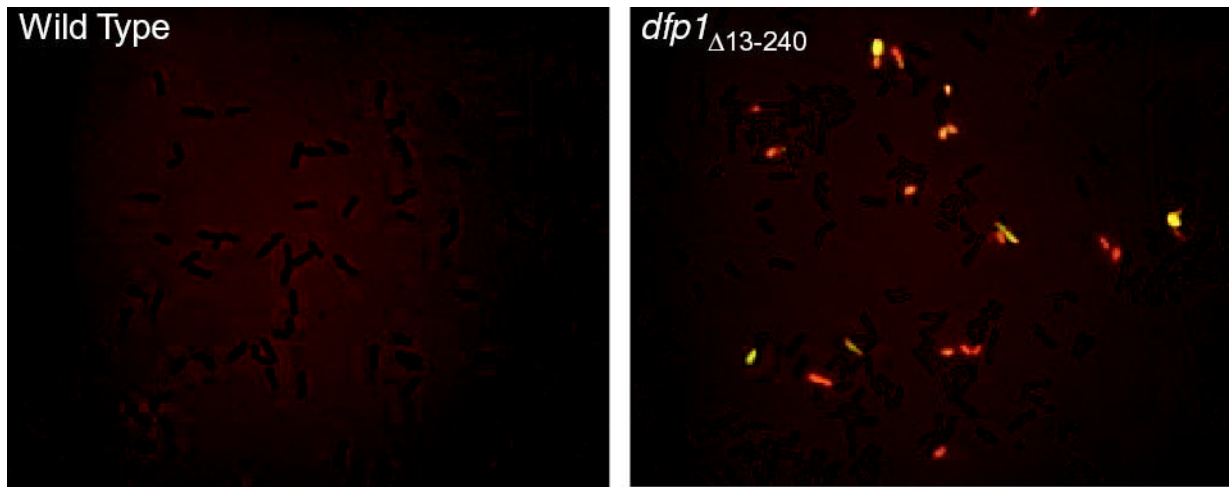
### References

- Al-Khodairy F, Fotou E, Sheldrick KS, Griffiths DJ, Lehmann AR, Carr AM. Identification and characterization of new elements involved in checkpoint and feedback controls in fission yeast. *Mol Biol Cell* 1994;5:147–160. [PubMed: 8019001]
- Balzan R, Sapienza K, Galea DR, Vassallo N, Frey H, Bannister WH. Aspirin commits yeast cells to apoptosis depending on carbon source. *Microbiology* 2004;150:109–115. [PubMed: 14702403]
- Bell SP, Dutta A. DNA replication in eukaryotic cells. *Annu Rev Biochem* 2002;71:333–374. [PubMed: 12045100]
- Bentley NJ, Holtzman DA, Flaggs G, Keegan KS, DeMaggio A, Ford JC, Hoekstra M, Carr AM. The *Schizosaccharomyces pombe rad3* checkpoint gene. *The EMBO Journal* 1996;15:6641–6651. [PubMed: 8978690]
- Blanchard F, Rusiniak ME, Sharma K, Sun X, Todorov I, Castellano MM, Gutierrez C, Baumann H, Burhans WC. Targeted destruction of DNA replication protein Cdc6 by cell death pathways in mammals and yeast. *Mol Biol Cell* 2002;13:1536–1549. [PubMed: 12006651]
- Boddy MN, Furnari B, Mondesert O, Russell P. Replication checkpoint enforced by kinases Cds1 and Chk1. *Science* 1998;280:909–912. [PubMed: 9572736]
- Brezniceanu ML, Völp K, Bösser S, Solbach C, Lichter P, Joos S, Zörnig M. HMGB1 inhibits cell death in yeast and mammalian cells and is abundantly expressed in human breast carcinoma. *FASEB J* 2003;17:1295–1297. [PubMed: 12759333]
- Burhans WC, Weinberger M, Marchetti MA, Ramachandran L, D'Urso G, Huberman JA. Apoptosis-like yeast cell death in response to DNA damage and replication defects. *Mutation Research* 2003;532:227–243. [PubMed: 14643439]
- Caspari T, Carr AM. DNA structure checkpoint pathways in *Schizosaccharomyces pombe*. *Biochimie* 1999;81:173–181. [PubMed: 10214922]
- Castedo M, Perfettini JL, Roumier T, Andreau K, Medema R, Kroemer G. Cell death by mitotic catastrophe: a molecular definition. *Oncogene* 2004;23:2825–2837. [PubMed: 15077146]

- Castedo M, Perfettini JL, Roumier T, Kroemer G. Cyclin-dependent kinase-1: linking apoptosis to cell cycle and mitotic catastrophe. *Cell Death Differ* 2002;9:1287–1293. [PubMed: 12478465]
- Chahwan C, Nakamura TM, Sivakumar S, Russell P, Rhind N. The fission yeast Rad32 (Mre11)-Rad50-Nbs1 complex is required for the S-phase DNA damage checkpoint. *Mol Cell Biol* 2003;23:6564–6573. [PubMed: 12944482]
- Cheng J, Park TS, Chio LC, Fischl AS, Yi XS. Induction of apoptosis by sphingoid long-chain bases in *Aspergillus nidulans*. *Mol Cell Biol* 2003;23:163–177. [PubMed: 12482970]
- Dodson GE, Shi Y, Tibbetts RS. DNA replication defects, spontaneous DNA damage, and ATM-dependent checkpoint activation in replication protein A-deficient cells. *J Biol Chem* 2004;279:34010–34014. [PubMed: 15197179]
- Duncker BP, Brown GW. Cdc7 kinases (DDKs) and checkpoint responses: lessons from two yeasts. *Mutation Research* 2003;532:21–27. [PubMed: 14643426]
- Feng D, Tu Z, Wu W, Liang C. Inhibiting the expression of DNA replication-initiation proteins induces apoptosis in human cancer cells. *Cancer Res* 2003;63:7356–7364. [PubMed: 14612534]
- Fleury C, Mignotte B, Vayssière JL. Mitochondrial reactive oxygen species in cell death signaling. *Biochimie* 2002;84:131–141. [PubMed: 12022944]
- Fung AD, Ou J, Bueler S, Brown GW. A conserved domain of *Schizosaccharomyces pombe* *dfp1*<sup>+</sup> is uniquely required for chromosome stability following alkylation damage during S phase. *Mol Cell Biol* 2002;22:4477–4490. [PubMed: 12052858]
- Furuya K, Carr AM. DNA checkpoints in fission yeast. *J Cell Sci* 2003;116:3847–3848. [PubMed: 12953049]
- Gould KL, Nurse P. Tyrosine phosphorylation of the fission yeast *cdc2*<sup>+</sup> protein kinase regulates entry into mitosis. *Nature* 1989;342:39. [PubMed: 2682257]
- Hartsuiker E, Vaessen E, Carr AM, Kohli J. Fission yeast Rad50 stimulates sister chromatid recombination and links cohesion with repair. *The EMBO Journal* 2001;20:6660–6671. [PubMed: 11726502]
- James C, Gschmeissner S, Fraser A, Evan GI. CED-4 induces chromatin condensation in *Schizosaccharomyces pombe* and is inhibited by direct physical association with CED-9. *Curr Biol* 1997;7:246–252. [PubMed: 9094313]
- Jimenez G, Yucel J, Rowley R, Subramani S. The *rad3*<sup>+</sup> gene of *Schizosaccharomyces pombe* is involved in multiple checkpoint functions and in DNA repair. *Proceedings of the National Academy of Sciences USA* 1992;89:4952–4956.
- Jürgensmeier JM, Krajewski S, Armstrong RC, Wilson GM, Oltersdorf T, Fritz LC, Reed JC, Otilie S. Bax- and Bak-induced cell death in the fission yeast *Schizosaccharomyces pombe*. *Mol Biol Cell* 1997;8:325–339. [PubMed: 9190211]
- Kelly TJ, Martin GS, Forsburg SL, Stephen RJ, Russo A, Nurse P. The fission yeast *cdc18*<sup>+</sup> gene product couples S phase to START and mitosis. *Cell* 1993;74:371–382. [PubMed: 7916658]
- Kiely J, Haase SB, Russell P, Leatherwood J. Functions of fission yeast Orp2 in DNA replication and checkpoint control. *Genetics* 2000;154:599–607. [PubMed: 10655214]
- Kim JM, Yamada M, Masai H. Functions of mammalian Cdc7 kinase in initiation/monitoring of DNA replication and development. *Mutation Research* 2003;532:29–40. [PubMed: 14643427]
- Leatherwood J, Lopez-Girona A, Russell P. Interaction of Cdc2 and Cdc18 with a fission yeast ORC2-like protein. *Nature* 1996;379:360–363. [PubMed: 8552194]
- LeBel CP, Ischiropoulos H, Bondy SC. Evaluation of the probe 2',7'-dichlorofluorescein as an indicator of reactive oxygen species formation and oxidative stress. *Chem Res Toxicol* 1992;5:227–231. [PubMed: 1322737]
- Lindsay HD, Griffiths DJF, Edwards RJ, Christensen PU, Murray JM, Osman F, Walworth N, Carr AM. S-phase-specific activation of Cds1 kinase defines a subpathway of the checkpoint response in *Schizosaccharomyces pombe*. *Genes Dev* 1998;12:382–395. [PubMed: 9450932]
- Madeo F, Fröhlich E, Ligr M, Grey M, Sigrist SJ, Wolf DH, Fröhlich KU. Oxygen stress: a regulator of apoptosis in yeast. *J Cell Biol* 1999;145:757–767. [PubMed: 10330404]
- Madeo F, Herker E, Wissing S, Jungwirth H, Eisenberg T, Fröhlich KU. Apoptosis in yeast. *Curr Opin Microbiol* 2004;7:655–660.

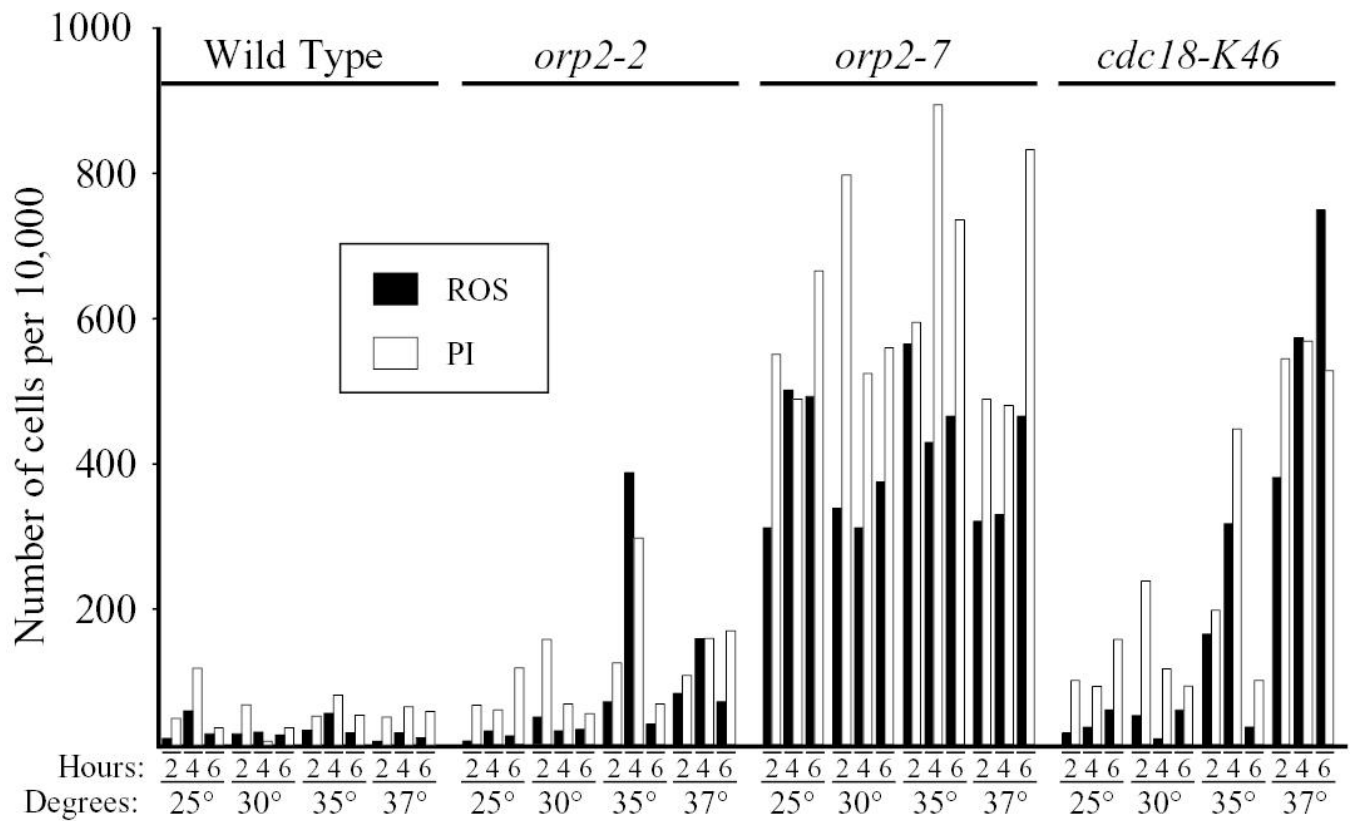
- Maftahi M, Hope JC, Delgado-Cruzata L, Han CS, Freyer GA. The severe slow growth of  $\Delta srs2 \Delta rqh1$  in *Schizosaccharomyces pombe* is suppressed by loss of recombination and checkpoint genes. *Nucleic Acids Research* 2002;30:4781–4792. [PubMed: 12409469]
- Manolis KG, Nimmo ER, Hartsuiker E, Carr AM, Jeggo PA, Allshire RC. Novel functional requirements for non-homologous DNA and joining in *Schizosaccharomyces pombe*. *The EMBO Journal* 2001;20:210–221. [PubMed: 11226171]
- Marchetti MA, Kumar S, Hartsuiker E, Maftahi M, Carr AM, Freyer GA, Burhans WC, Huberman JA. A single unbranched S-phase DNA damage and replication fork blockage checkpoint pathway. *Proceedings of the National Academy of Sciences USA* 2002;99:7472–7477.
- Mizukami T, Chang WI, Garkavtsev I, Kaplan N, Lombardi D, Matsumoto T, Niwa O, Kounosu A, Yanagida M, Marr TG, et al. A 13 kb resolution cosmid map of the 14 Mb fission yeast genome by nonrandom sequence-tagged site mapping. *Cell* 1993;73:121–132. [PubMed: 8462095]
- Moreno S, Klar A, Nurse P. Molecular genetic analysis of fission yeast, *Schizosaccharomyces pombe*. *Methods Enzymol* 1991;194:795–823. [PubMed: 2005825]
- Murakami H, Okayama H. A kinase from fission yeast responsible for blocking mitosis in S phase. *Nature* 1995;374:817–819. [PubMed: 7723827]
- Muzi-Falconi M, Brown GW, Kelly TJ. *cdc18<sup>+</sup>* regulates initiation of DNA replication in *Schizosaccharomyces pombe*. *Proceedings of the National Academy of Sciences USA* 1996;93:1566–1570.
- Norbury CJ, Zhitovovskiy B. DNA damage-induced apoptosis. *Oncogene* 2004;23:2797–2808. [PubMed: 15077143]
- Nyberg KA, Michelson RJ, Putnam CW, Weinert TA. TOWARD MAINTAINING THE GENOME: DNA damage and replication checkpoints. *Annu Rev Genet* 2002;36:617–656. [PubMed: 12429704]
- Ogino K, Takeda T, Matsui E, Iiyama H, Taniyama C, Arai K-i, Masai H. Bipartite binding of a kinase activator activates Cdc7-related kinase essential for S phase. *J Biol Chem* 2001;276:31376–31387. [PubMed: 11402029]
- Pelizon C, d'Adda di Fagagna F, Farrace L, Laskey RA. Human replication protein Cdc6 is selectively cleaved by caspase during apoptosis. *EMBO Rep* 2002;3:780–784. [PubMed: 12151338]
- Qi H, Li TK, Kuo D, Nur-E-Kamal A, Liu LF. Inactivation of Cdc13p triggers *MEC1*-dependent apoptotic signals in yeast. *J Biol Chem* 2003;278:15136–15141. [PubMed: 12569108]
- Rhind N, Russell P. Checkpoints: It takes more than time to heal some wounds. *Curr Biol* 2000;10:R908–R911. [PubMed: 11137027]
- Rodriguez-Menocal L, D'Urso G. Programmed cell death in fission yeast. *FEMS Yeast Research* 2004;5:111–117. [PubMed: 15489193]
- Sancar A, Lindsey-Boltz LA, Unsal-Kacmaz K, Linn S. Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. *Annu Rev Biochem* 2004;73:39–85. [PubMed: 15189136]
- Schories B, Engel K, Dorken B, Gossen M, Bommert K. Characterization of apoptosis-induced Mcm3 and Cdc6 cleavage reveals a proapoptotic effect for one Mcm3 fragment. *Cell Death Differ* 2004;11:940–942. [PubMed: 15002044]
- Shreeram S, Sparks A, Lane DP, Blow JJ. Cell type-specific responses of human cells to inhibition of replication licensing. *Oncogene* 2002;21:6624–6632. [PubMed: 12242660]
- Takeda T, Ogino K, Matsui E, Cho MK, Kumagai H, Miyake T, Arai K-i, Masai H. A fission yeast gene, *him1<sup>+</sup>/dfp1<sup>+</sup>*, encoding a regulatory subunit for Hsk1 kinase, plays essential roles in S-phase initiation as well as in S-phase checkpoint control and recovery from DNA damage. *Mol Cell Biol* 1999;19:5535–5547. [PubMed: 10409743]
- Tavassoli M, Shayeghi M, Nasim A, Watts FZ. Cloning and characterisation of the *Schizosaccharomyces pombe rad32* gene: a gene required for repair of double strand breaks and recombination. *Nucleic Acids Research* 1995;23:383–388. [PubMed: 7885834]
- Tsuchiya M, Suematsu M, Suzuki H. In vivo visualization of oxygen radical-dependent photoemission. *Methods Enzymol* 1994;233:128–140. [PubMed: 8015451]
- Venkitaraman AR. Aborting the birth of cancer. *Nature* 2005;434:829–830. [PubMed: 15829943]
- Walworth N, Davey S, Beach D. Fission yeast *chk1* protein kinase links the *rad* checkpoint pathway to *cdc2*. *Nature* 1993;363:368–371. [PubMed: 8497322]

- Weinberger, M., Ramachandran, L., Feng, L., Sharma, K., Sun, X., Marchetti, M. A., Huberman, J. A. and Burhans, W. C. (2005).** Apoptosis in budding yeast caused by defects in initiation of DNA replication. *J. Cell Sci.* In press.
- Willson J, Wilson S, Warr N, Watts FZ. Isolation and characterization of the *Schizosaccharomyces pombe* *rhp9* gene: a gene required for the DNA damage checkpoint but not the replication checkpoint. *Nucleic Acids Research* 1997;25:2138–2145. [PubMed: 9153313]
- Yim H, Jin YH, Park BD, Choi HJ, Lee SK. Caspase-3-mediated cleavage of Cdc6 induces nuclear localization of p49-truncated Cdc6 and apoptosis. *Mol Biol Cell* 2003;14:4250–4259. [PubMed: 14517333]
- Zhang Q, Chieu HK, Low CP, Zhang S, Heng CK, Yang H. *Schizosaccharomyces pombe* cells deficient in triacylglycerols synthesis undergo apoptosis upon entry into the stationary phase. *J Biol Chem* 2003;278:47145–47155. [PubMed: 12963726]

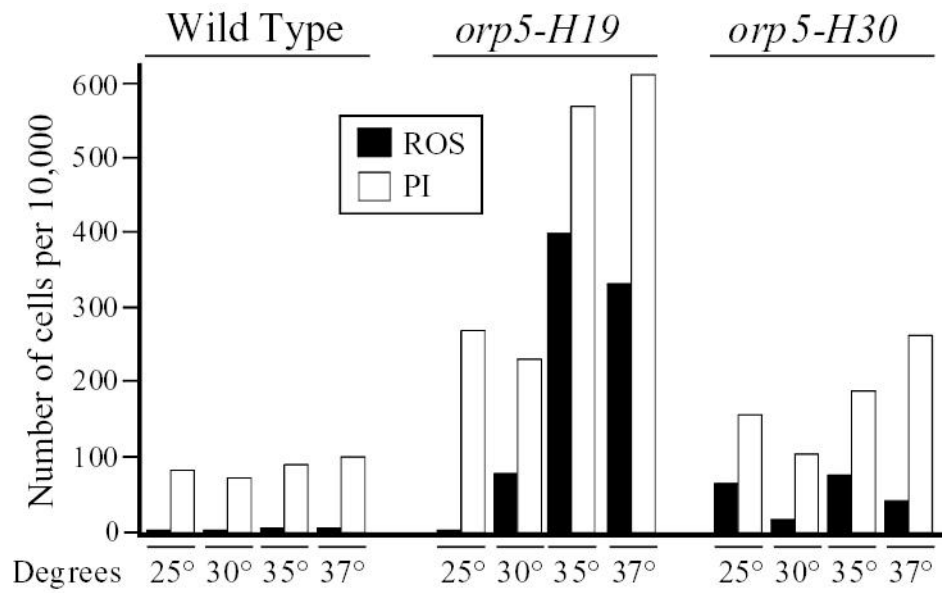


**Fig. 1.** Detection of ROS production and death in fission yeast cells. Wild type and *dfp1* $\Delta_{13-240}$  mutant cells were incubated for 80 minutes at 25°C in the presence of DCDHFDA to detect ROS. The cells were then harvested, washed, and resuspended in buffer with PI to detect dead cells.

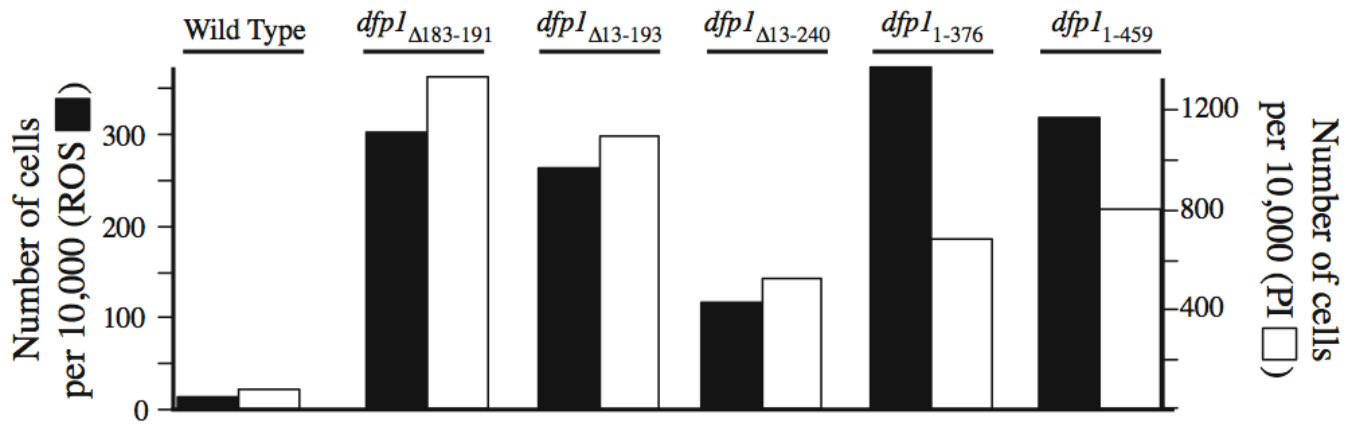


**Fig. 2.**

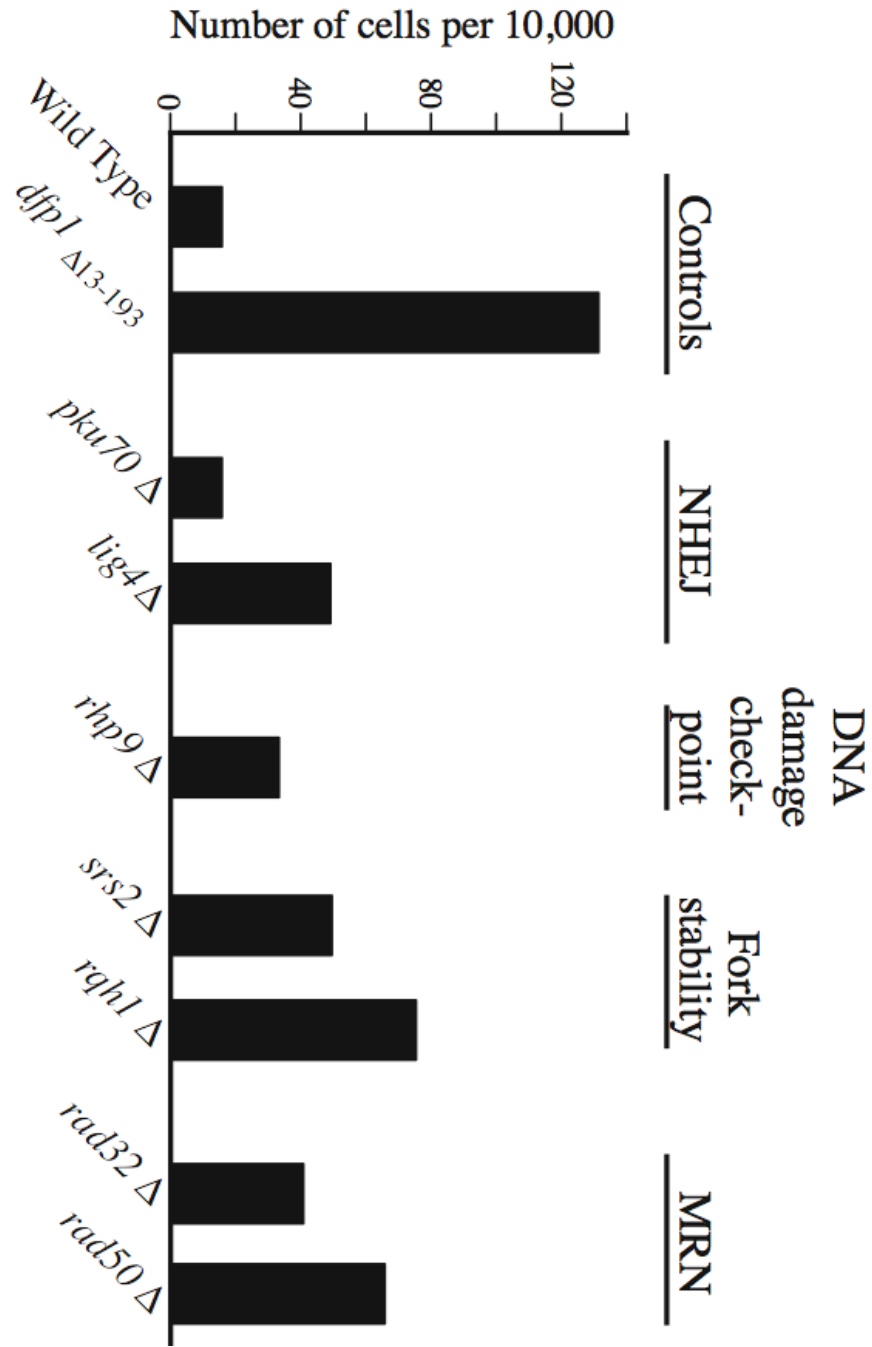
A time- and temperature-dependence study of ROS production and PI staining in cells bearing temperature-sensitive mutations in *orp2* or *cdc18*. The indicated strains, in log phase, were shifted to the indicated temperatures for the indicated numbers of hours. Then DCDHFDA was added, and incubation was continued at the same temperatures for an additional 80 minutes. Other conditions were as in Figs. 1 and S1.



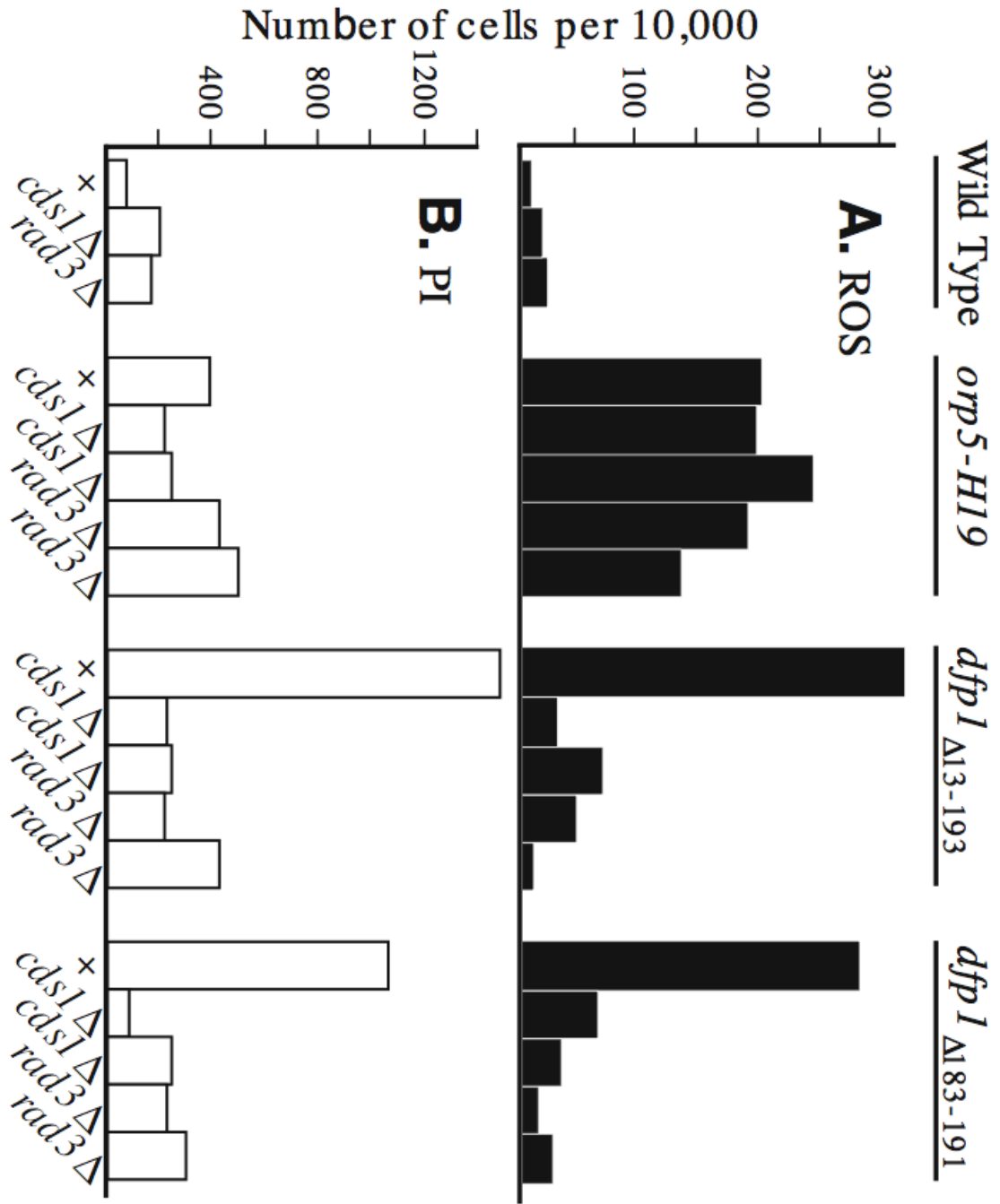
**Fig. 3.** A temperature-dependence study of ROS production and PI staining in cells bearing temperature-sensitive mutations in *orp5*. This experiment was carried out as in Fig. 2, except that the time of incubation was 4 hours in all cases.



**Fig. 4.** N- and C-terminal deletions in *dfp1* lead to ROS production and PI staining. The indicated fission yeast strains were incubated for 80 minutes at 25°C in the presence of DCDHFDA and also stained with PI as in Fig. 1.

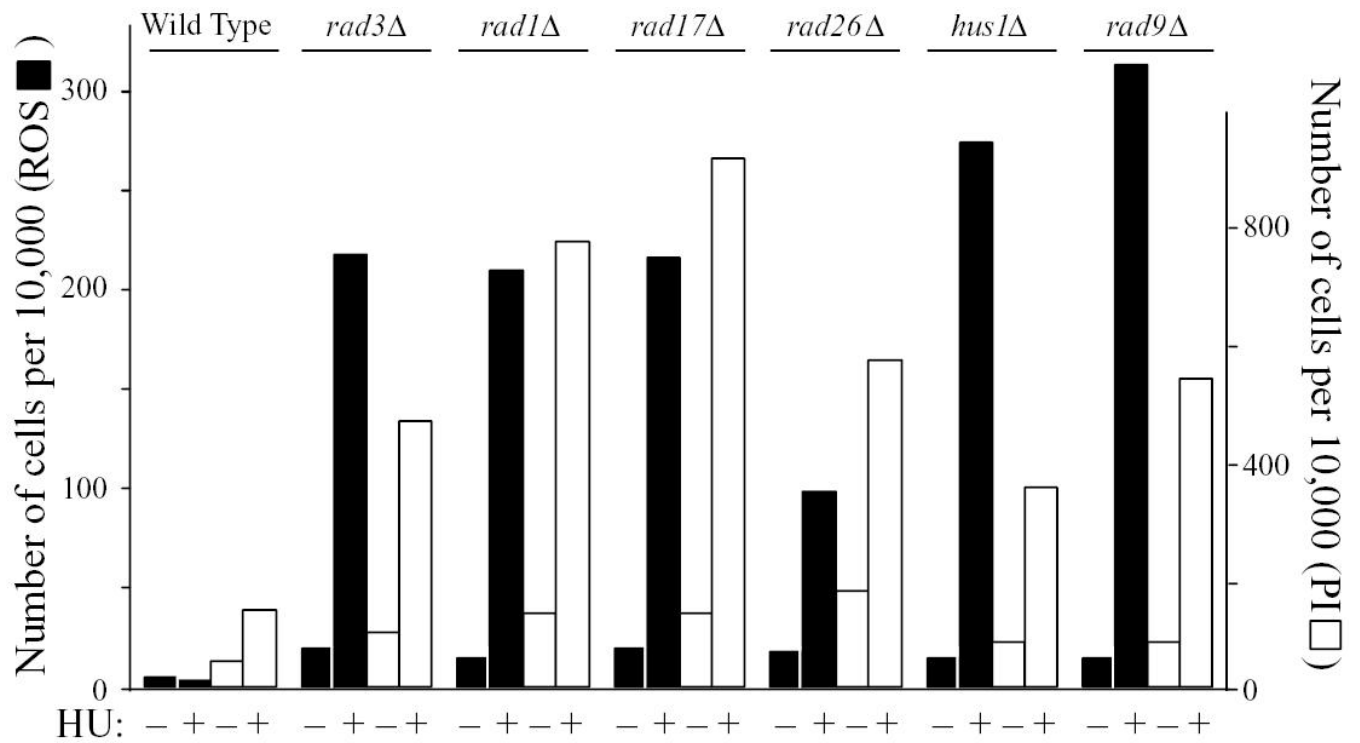


**Fig. 5.** Mutants in pathways affecting non-homologous end joining, the DNA damage checkpoint, replication fork stability, and the MRN complex appear to produce less ROS than mutants in replication proteins. The indicated strains were incubated at 25°C for 80 minutes in the presence of DCDHFDA. Other conditions were as in Figs. 1 and 4.

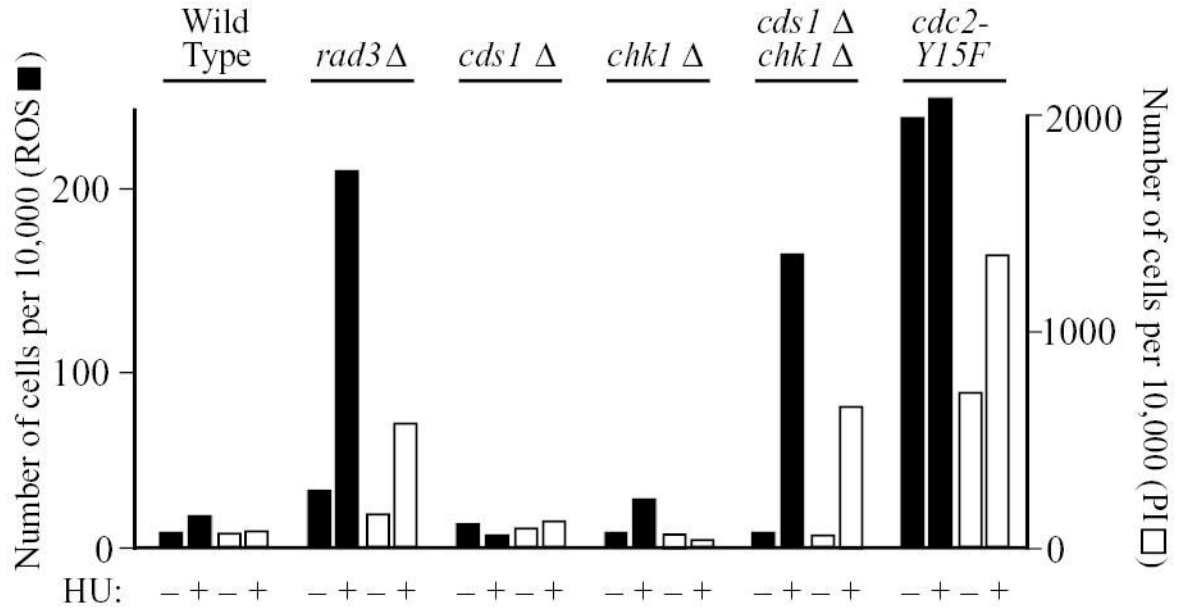


**Fig. 6.** ROS production by *dfp1* N-terminal deletion mutants requires a functional Rad3p- and Cds1p-dependent checkpoint pathway, but ROS production by the *orp5-H19* strain does not. Double-mutant strains bearing *orp5-H19* or *dfp1* Δ13-193 or *dfp1* Δ183-191 along with *rad3* Δ or *cds1* Δ were generated by crossing. Results are shown for two independently derived isolates of each type. Strains lacking a *cds1* Δ or *rad3* Δ mutation are indicated by “+”. Cells in early log phase were incubated at 30°C for six hours. (A) ROS production. (B) PI staining.

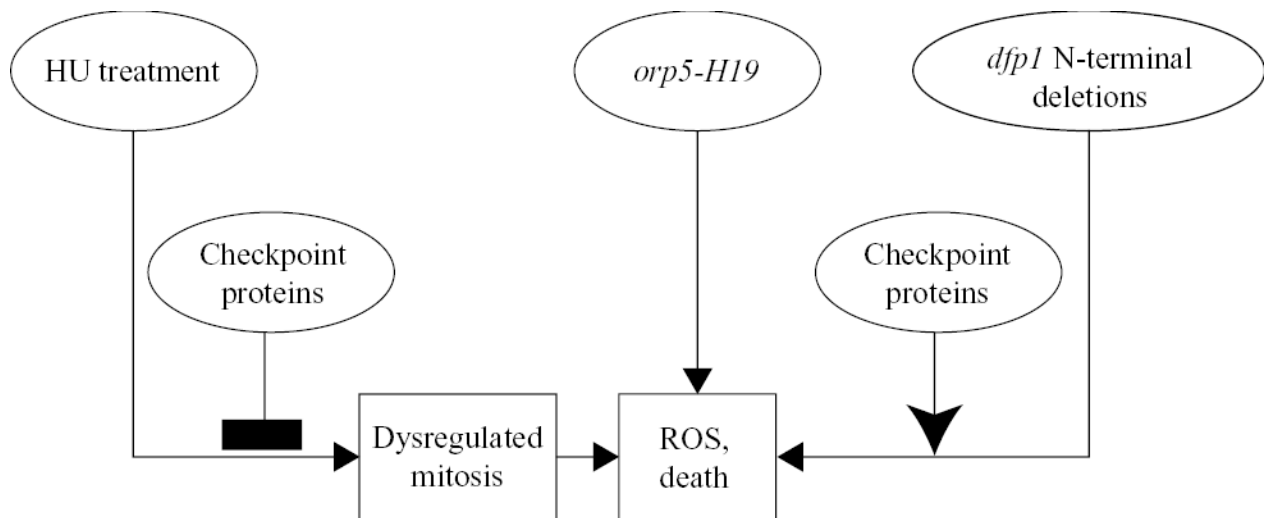




**Fig. 7.** Deletion of any of the checkpoint-Rad genes leads to elevated ROS production and PI staining in the presence of HU. The indicated strains were incubated at 25°C for 4 hours with or without 12 mM HU. Then DCDHFDA was added, and incubation was continued for 80 minutes. The cells were stained with PI and analyzed by flow cytometry as in previous figures.



**Fig. 8.** Stimulation of ROS production and PI staining by HU requires abrogation of both the Cds1p- and Chk1p-dependent checkpoint pathways or dysregulation of Cdc2p. The indicated strains were incubated at 25°C for 4 hours with or without 12 mM HU. Then DCDHFDA was added, and incubation was continued for 80 minutes. The cells were stained with PI and analyzed by flow cytometry as in previous figures.

**Fig. 9.**

Checkpoint-inhibited, checkpoint-stimulated, and checkpoint-independent pathways leading to production of ROS and cell death in fission yeast. This figure provides a simplified diagrammatic summary of the pathways leading from mutations in genes encoding replication initiation proteins, or from HU treatment, to elevated ROS production and death.

**Table 1**

Names of relevant functionally similar genes in fission yeast, budding yeast, and humans

<b>Fission yeast</b>	<b>Humans</b>	<b>Budding yeast</b>	<b>Protein function</b>
<i>dfp1</i>	<i>DBF4/ASK1</i>	<i>DBF4</i>	Initiation of replication
<i>hsk1</i>	<i>CDC7</i>	<i>CDC7</i>	Initiation of replication
<i>cdc18</i>	<i>CDC6</i>	<i>CDC6</i>	Formation of pre-replication complex
<i>orp2</i>	<i>ORC2</i>	<i>ORC2</i>	Formation of pre-replication complex
<i>orp5</i>	<i>ORC5</i>	<i>ORC5</i>	Formation of pre-replication complex
<i>pku70</i>	<i>KU70</i>	<i>YKU70</i>	Non-homologous end joining
<i>rad32</i>	<i>MRE11</i>	<i>MRE11</i>	Homologous recombination
<i>rqh1</i>	<i>BLM</i>	<i>SGS1</i>	Replication fork stability
<i>rhp9/crb2</i>	<i>BRCA1</i> or <i>53BP1</i>	<i>RAD9</i>	DNA damage checkpoint
<i>rad3</i>	<i>ATR</i>	<i>MEC1</i>	DNA damage and replication checkpoints
<i>cds1</i>	<i>CHK2/CDS1</i>	<i>RAD53</i>	DNA replication checkpoint (fission yeast)
<i>cdc2</i>	<i>CDK1/CDC2</i>	<i>CDC28</i>	Cyclin-dependent kinase

This table contains the genes discussed in the text for which the fission yeast name differs from the human name.