

Apoptosis in budding yeast caused by defects in initiation of DNA replication

Martin Weinberger¹, Lakshmi Ramachandran¹, Li Feng¹, Karuna Sharma², Xiaolei Sun¹, Maria Marchetti², Joel A. Huberman² and William C. Burhans^{1,*}

¹Department of Cell Stress Biology, Roswell Park Cancer Institute, Buffalo, NY 14263, USA

²Department of Cancer Genetics, Roswell Park Cancer Institute, Buffalo, NY 14263, USA

*Author for correspondence (e-mail: wburhans@acsu.buffalo.edu)

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Summary

Apoptosis in metazoans is often accompanied by the destruction of DNA replication initiation proteins, inactivation of checkpoints and activation of cyclin-dependent kinases, which are inhibited by checkpoints that directly or indirectly require initiation proteins. Here we show that, in the budding yeast *Saccharomyces cerevisiae*, mutations in initiation proteins that attenuate both the initiation of DNA replication and checkpoints also induce features of apoptosis similar to those observed in metazoans. The apoptosis-like phenotype of initiation mutants includes the production of reactive oxygen species (ROS) and activation of the budding-yeast metacaspase Yca1p. In contrast to a recent report that activation of Yca1p only occurs in lysed cells and does not contribute to cell death, we found that, in at least one initiation mutant, Yca1p activation occurs at an early stage of cell death

(before cell lysis) and contributes to the lethal effects of the mutation harbored by this strain. Apoptosis in initiation mutants is probably caused by DNA damage associated with the combined effects of insufficient DNA replication forks to completely replicate the genome and defective checkpoints that depend on initiation proteins and/or replication forks to restrain subsequent cell-cycle events until DNA replication is complete. A similar mechanism might underlie the proapoptotic effects associated with the destruction of initiation and checkpoint proteins during apoptosis in mammals, as well as genome instability in initiation mutants of budding yeast.

Key words: Apoptosis, Initiation of DNA replication, Checkpoints, Metacaspase

Introduction

In eukaryotic cells, DNA replication is coupled to the cell-division cycle by the reciprocal regulation of proteins required for initiation at origins of DNA replication and molecular events regulated by cyclin-dependent kinases (CDKs) that drive the cell cycle forward. Origins of replication are 'licensed' for activation in S phase by the assembly in G1 of prereplicative complexes (pre-RCs) containing the origin recognition complex (ORC) and other proteins, including the ORC-interacting initiation protein Cdc6. To ensure that replication of chromosomes occurs just once each cell cycle, activation of origins in S phase coincides with the inactivation and disassembly of pre-RCs, and CDKs and other proteins block their reassembly until the end of mitosis, when CDK activity is destroyed (for a review, see Diffley, 2001). In G1 and early S phase cells, CDKs and/or CDK-dependent molecular events required for completion of S phase and mitosis are, in turn, inhibited by checkpoints that depend on pre-RC proteins, either directly (Clay-Farrace et al., 2003; Oehlmann et al., 2004; Toyn et al., 1995; Weinreich et al., 2001) or indirectly through the establishment of DNA replication forks (Tercero et al., 2003). The reciprocal regulation of pre-RCs, CDKs and/or CDK-dependent events couples DNA replication to the cell cycle, providing a stringent, fail-safe mechanism for ensuring that a single but complete copy of the genome is produced each time a cell divides.

Uncoupling of DNA replication from the cell-division cycle might be an important feature of apoptosis. This was first suggested by similarities between apoptosis and mitotic catastrophe in cells with incompletely replicated DNA (for review, see King and Cidlowski, 1995). Apoptosis also coincides with the destruction of the pre-RC proteins Cdc6 (Blanchard et al., 2002; Burhans et al., 2002; Illenye and Heintz, 2004; Pelizon et al., 2002; Schories et al., 2004; Yim et al., 2003; Yin et al., 2004) and Mcm3 (Schwab et al., 1998), the CDK inhibitors Wee1 (Zhou et al., 1998), p21 and p27 (Levkau et al., 1998) (for a review, see Gartel and Tyner, 2002), and the checkpoint proteins ATM (Smith et al., 1999), Rb (Tan et al., 1997) and Rad9 (Lee et al., 2003). The destruction of Cdc6, p21, Rad9 and Rb contributes to apoptosis because cells can be rescued from apoptotic stimuli, downstream of the activation of caspases, by expressing mutant versions of these proteins that cannot be cleaved by caspases (Gartel and Tyner, 2002; Huang et al., 1999; Lee et al., 2003; Pelizon et al., 2002; Tan et al., 1997; Yim et al., 2003). Apoptosis also often coincides with the premature activation of S phase and mitotic CDKs that are restrained by pre-RC-dependent and other checkpoints (for reviews, see Castedo et al., 2002; Guo and Hay, 1999; King and Cidlowski, 1995; Meikrantz and Schlegel, 1995). In addition to the attenuation of apoptosis that occurs when destruction of CDK inhibitors are blocked, apoptosis is also attenuated when CDK activation is directly inhibited by,

for example, dominant-negative CDK mutants (for reviews, see Castedo et al., 2002; Gartel and Tyner, 2002; Guo and Hay, 1999). All these findings indicate that uncoupling DNA replication from the cell cycle by events that simultaneously inhibit initiation of DNA replication and abrogate checkpoints can be an important component of a commitment to cell death during apoptosis.

Several features of apoptosis that were detected in metazoans have also been detected in yeasts, including the production of reactive oxygen species (ROS) and the activation of a recently discovered metacaspase, Yca1p (for reviews, see Burhans et al., 2003; Madeo et al., 2002a; Weinberger et al., 2003). Here, we show that, in budding yeast, the temperature-sensitive *orc2-1* mutation, which uncouples DNA replication from the cell cycle by simultaneously inhibiting pre-RC assembly (Santocanale and Diffley, 1996) and checkpoints that depend on DNA replication forks to restrain subsequent cell-cycle events (Shimada et al., 2002; Shirahige et al., 1998b; Weinberger et al., 1999) induces ROS and activation of Yca1p coincidentally with the activation of a Rad53p-dependent response to DNA damage. In contrast to a recent report that Yca1p activation signals are not associated with lethality and only occur in lysed cells (Wysocki and Kron, 2004), activation of Yca1p by the *orc2-1* mutation was detected in cells with intact membranes and contributed to the lethal effects of this mutation. The results of our experiments suggest that uncoupling of DNA replication from the cell cycle contributes to apoptosis by inducing DNA damage. They also provide definitive evidence that, similar to mammals, DNA damage can induce apoptosis in budding yeast and emphasize the important role that the Yca1p metacaspase plays in budding yeast apoptotic programs.

Materials and Methods

Cell culture, apoptosis detection and DNA staining

Standard protocols for growth of yeast were used (Guthrie and Fink, 1991). Cells were grown in YPD except where plasmid maintenance required auxotrophic selection in defined medium, and YP-galactose was used to induce galactose-regulated gene expression. Cell cultures were maintained at an early to mid-log-phase state of growth. ROS and propidium iodide (PI) were detected by flow cytometry (FACS) using 2',7'-dichlorodihydrofluorescein diacetate (H₂DCF-DA, Molecular Probes) to detect ROS as described in (Madeo et al., 1997) and a Becton Dickinson FACScan equipped with a 488 nm argon laser. Emission signals were detected at 525–550 nm. Before FACS analysis, cells were resuspended in 50 mM sodium citrate containing 1 µg ml⁻¹ PI (Sigma). Caspase activity was detected using FITC-VAD-FMK (Promega) according to the manufacturer's instructions. In brief, 5 × 10⁶ cells were stained with 100 µM FITC-VAD-FMK and 1 µg ml⁻¹ PI at room temperature in the dark for 20 minutes. They were then washed and resuspended in 1 × PBS before analysis by FACS at an excitation wavelength of 488 nm and emission wavelength of 525–550 nm. Effects of a *YCA1* deletion (*yca1Δ*) on viability were determined by colony-forming assays. Growth in rich medium with proper aeration was crucial for optimal detection of ROS and activated caspase signals, and for detecting the effects of deleting the *YCA1* gene on viability. In general, growth in minimal medium (which was required for experiments involving ectopic expression of genes from plasmids) reduced the frequency with which ROS and activated caspase signals were detected compared with growth in rich medium. FACS measurements of DNA content were as described previously (Weinberger et al., 1999), except that Sytox Green (Molecular Probes) was used instead of PI. DAPI staining of cell nuclei was as described

previously (Guthrie and Fink, 1991). FACS data were processed and quantified using Cell Quest (Becton Dickinson) and WinList (Verity) software. Values presented in graphs are means of three or more independent experiments or are representative of similar results in multiple independent experiments. Photomicrographs of stained cells were obtained at 400× magnification with a Zeiss Axioskop microscope equipped with an Optronics Magnafire CCD camera.

Random spore analysis, cell-cycle viability and arrest and inhibitors

Standard protocols for mating and random spore analysis were used (Guthrie and Fink, 1991). Cell-viability experiments involving temperature shifts, drug treatments and cell-cycle arrests were as previously described (Weinberger et al., 1999). The initial G1-phase arrest in cell-staging experiments was at 23°C with 12 µM α mating factor (*orc2-1* strains) or 6 µM α factor (all other strains). After 2.5 hours, an equivalent amount of α factor was added and aliquots of the G1 arrested cultures were incubated for an additional 3 hours at 23°C and 35°C. Each G1-phase-arrested culture was then split into three additional aliquots. The G1-phase arrest was maintained in one culture and the others were released from G1 phase by filtering the culture at the same temperature as G1 arrest and resuspending cells in medium without α factor. All cultures were incubated for an additional 2 hours at the appropriate temperature before measuring cell viability. The proteasome inhibitor MG132 was added to a final concentration of 250 µM and the antioxidant *N*-acetyl-L-cysteine (NAC) was added to a final concentration of 4 mM.

Western blots

Crude lysates were prepared by bead beating in lysis buffer [100 mM NaCl, 25 mM Tris-HCl, 10% glycerol, 0.1% Triton X-100 with one protease inhibitor tablet (Roche) per 5 ml]. 30 µg protein were size fractionated on 12% PAGE gels and blotted proteins were visualized by immunodetection using an enhanced chemiluminescence (ECL) kit from Amersham. All antibodies were obtained from Santa Cruz Biotechnology.

Strains and plasmids and disruption of the *YCA1* gene

All strains were in the W303 background, and lists of strains and plasmids are provided in Table 2. *YCA1* was replaced in wild-type and *orc2-1* cells by standard techniques using a PCR-generated G418 resistance cassette (Bahler et al., 1998) and the following primers 5'-ACATAATAAATTGCAGATTTACGTCAATAGGGTGTGACGATG-ATAATTGTGGTTTTTGAGGAATTCGAGCTCGTTTAAAC-3' and 5'-ATGAAGATGAGCCTCGAAGTTTATCTAACTACCACCAA-GAAGACCGACTAGATTTACACGGATCCCGGGTTAATTA-3'. Confirmation of the disruption was obtained by Southern blots and sequence analysis.

Results

Induction of ROS at high temperatures by *orc2-1*

The temperature-sensitive *orc2-1* mutation causes lethality at nonpermissive temperatures for reasons that are not clear. Diploid *orc2-1* cells undergo DNA fragmentation and Rad9p-dependent cell death at higher temperatures (Watanabe et al., 2002), which suggests the induction of an apoptosis-like cell-death program. Consistent with this possibility, shifting GA1410 cells harboring the *orc2-1* mutation, but not isogenic wild-type cells, to 37°C induced another common feature of apoptosis, ROS. ROS were present in cells at several stages of the cell cycle, including S phase, as indicated by different bud

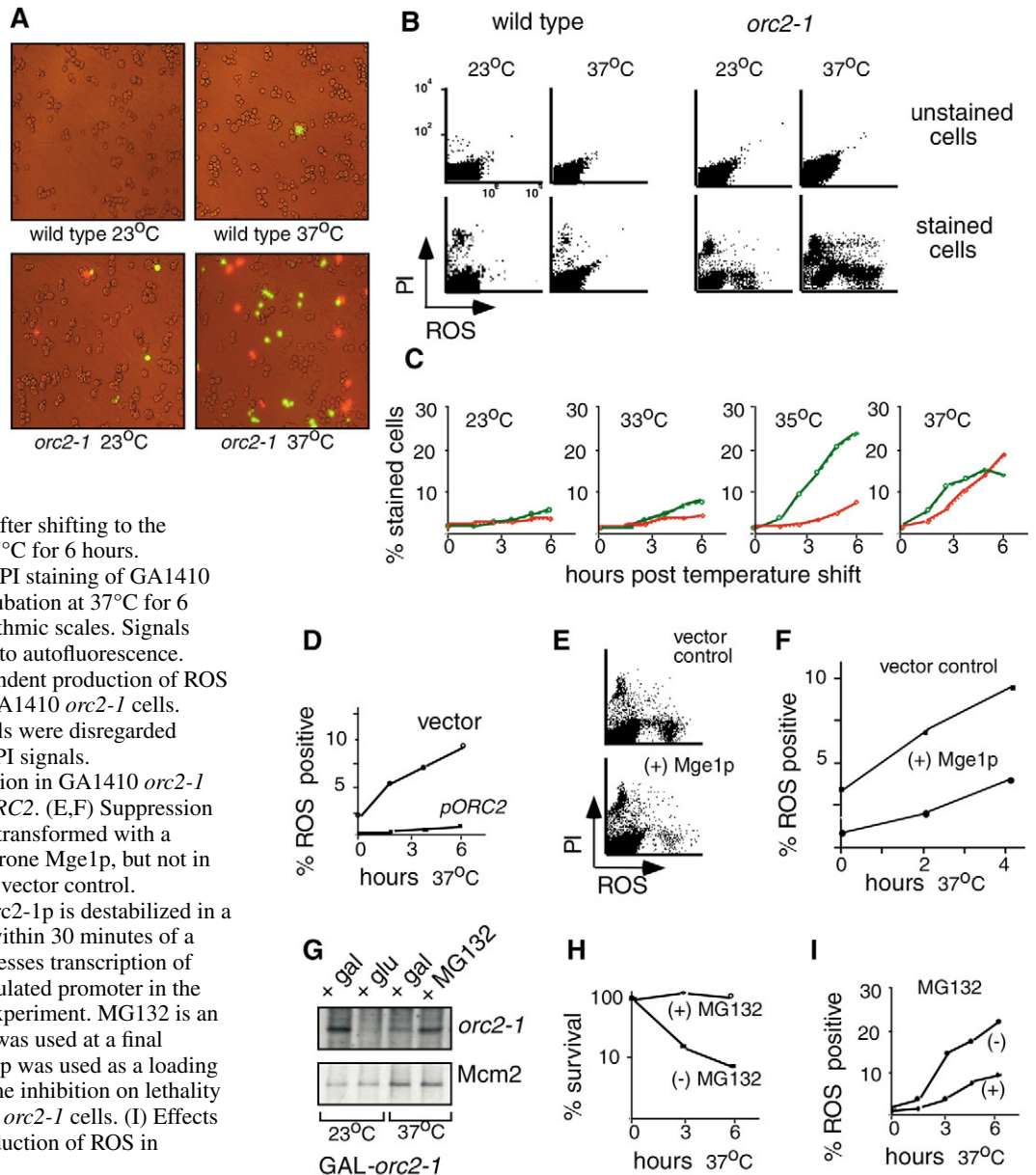


Fig. 1. Loss of Orc2p function leads to the production of ROS. (A) Wild-type (W303) and *orc2-1* (GA1410) cells stained with PI (red) and the ROS probe H₂DCF-DA (green) before and after shifting to the nonpermissive temperature of 37°C for 6 hours. (B) FACS detection of ROS and PI staining of GA1410 *orc2-1* cells before and after incubation at 37°C for 6 hours. Data are plotted on logarithmic scales. Signals from unstained cells correspond to autofluorescence. (C) Temperature- and time-dependent production of ROS (green) and PI (red) signals in GA1410 *orc2-1* cells. Signals from autofluorescing cells were disregarded during quantitation of ROS and PI signals. (D) Suppression of ROS production in GA1410 *orc2-1* cells by ectopic expression of *ORC2*. (E,F) Suppression of ROS in GA1410 *orc2-1* cells transformed with a plasmid expressing the co-chaperone Mge1p, but not in cells transformed with an empty vector control. (G) Western blot showing that *orc2-1p* is destabilized in a proteasome-dependent fashion within 30 minutes of a shift to 37°C. Glucose (glu) represses transcription of *orc2-1* driven by a galactose-regulated promoter in the GAL-*orc2-1* cells used in this experiment. MG132 is an inhibitor of the proteasome and was used at a final concentration of 250 μM. Mcm2p was used as a loading control. (H) Effects of proteasome inhibition on lethality of high temperatures in GA1410 *orc2-1* cells. (I) Effects of proteasome inhibition on production of ROS in GA1410 *orc2-1* cells.

sizes (Fig. 1A). Even at the semipermissive temperature of 23°C, at which ORC function is partly compromised in *orc2-1* cells (Santocane and Diffley, 1996), a several-hundredfold increase in numbers of ROS-producing cells was detected above the low (<0.1%) background levels of ROS-producing wild-type cells. FACS measurements of the number of ROS-positive *orc2-1* cells (Fig. 1B) showed that ROS production in these cells was temperature and time dependent (Fig. 1C). Parallel assessments of cell death detected by membrane changes that allow for staining of DNA in dead or dying cells by propidium iodide, a membrane-impermeable dye, indicated that both ROS production and cell death were rapid, and suggested that ROS production can precede loss of viability (Fig. 1C).

Induction of many ROS-producing cells by high temperatures depended on the temperature-sensitive *orc2-1* mutation in GA1410 cells, because production of ROS in these

cells was suppressed by ectopic expression of wild-type Orc2p (Fig. 1D), consistent with the recessive nature of the *orc2-1* mutation. ROS induced by the *orc2-1* mutation was also partly suppressed by ectopic expression of the co-chaperone protein Mge1p (Fig. 1E,F), which suppresses the initiation defect of *orc2-1* cells and stimulates initiation of DNA replication in wild-type cells (Trabold et al., 2005). At semipermissive temperatures, steady-state levels of *orc2-1p* are approximately ten times less than levels of Orc2p in wild-type cells, owing to the more rapid turnover of *orc2-1p* by the proteasome (Shimada et al., 2002). Shifting GAL-*orc2-1* cells to 37°C when grown in galactose to induce the production of high levels of *orc2-1p* from a galactose-inducible promoter (in order to more easily detect *orc2-1p*) also caused levels of *orc2-1p* to decline, but not when these cells were also treated with the proteasome inhibitor MG132 (Fig. 1G). Thus, higher temperatures accelerate the turnover of *orc2-1p* even further.

Inhibition of the proteasome by MG132 also rescued *orc2-1* cells from the lethal effects of high temperatures (Fig. 1H) and suppressed the production of ROS (Fig. 1I). Therefore, loss of viability and increased ROS at nonpermissive temperatures are related to acute loss of *orc2-1p*.

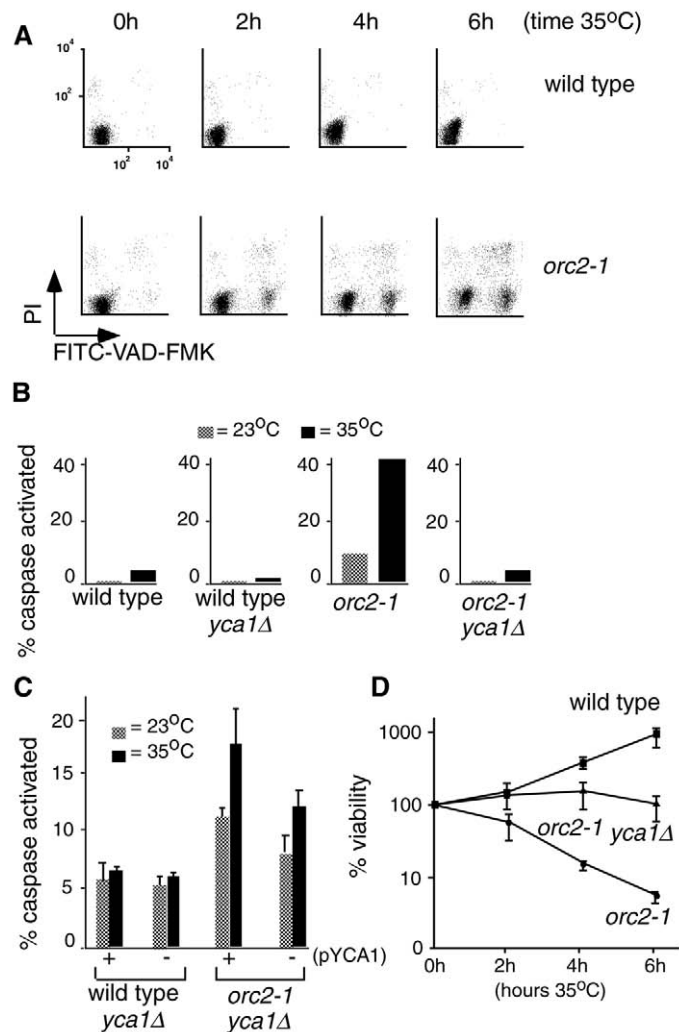


Fig. 2. Loss of Orc2p function activates the budding-yeast metacaspase Yca1p. (A) Detection of signals by FACS in wild-type and GA1410 *orc2-1* cells stained with propidium iodide (PI) and FITC-VAD-FMK, which stains cells containing activated caspases green. (B) The proportions of W303 wild-type and GA1410 *orc2-1* cells with caspase activity before and after shifting to 35°C for 6 hours in cells from which the *YCA1* metacaspase-encoding gene had (*yca1Δ*) or had not been deleted. (C) Detection of caspase activity in wild-type and GA1410 *orc2-1* cells with *YCA1* deleted, and transformed with a plasmid expressing Yca1p or an empty vector control plasmid. The increase in caspase activation in controls for this experiment compared with the experiment presented in B is caused by culturing cells in defined medium required for plasmid maintenance instead of rich medium (data not shown). (D) Effect of *yca1Δ* on viability of GA1410 *orc2-1* cells. Viability was measured as colony-forming units and was normalized to viability measured in cells maintained at 23°C compared with cells shifted to 35°C for indicated times.

Activation of the Yca1p metacaspase by *orc2-1*

Shifting GA1410 *orc2-1* cells to high temperatures also activated the recently discovered Yca1p metacaspase in budding yeast (Madeo et al., 2002b), as detected by a standard assay used in mammalian cells to detect activated caspases in cells undergoing apoptosis (Fig. 2). Substantially more cells with activated metacaspase (as indicated by staining with FITC-VAD-FMK) were detected in cultures of these *orc2-1* cells than in wild-type cells after a shift to the nonpermissive temperature of 35°C (Fig. 2A) [because ROS production was greatest at 35°C (Fig. 1C), Yca1p activation was measured after shifting to this temperature rather than 37°C]. Parallel incubation of these cells with PI indicated a much smaller increase in the number of cells that were stained with both PI and FITC-VAD-FMK (Fig. 2A). Therefore, most of the cells that were stained with FITC-VAD-FMK had not lost membrane integrity. This is in stark contrast to a recent report that the caspase activation signals detected by this assay in yeast cells are an artefact related to cell lysis (Wysocki and Kron, 2004). Furthermore, the number of cells with activated caspase signals was substantially reduced in *orc2-1* cells with *YCA1* deleted (Fig. 2B). In addition, partial restoration of caspase activation signals occurred in *orc2-1 yca1Δ* cells, but not in wild-type cells expressing Yca1p ectopically from a plasmid (Fig. 2C). These results unambiguously establish that activation of Yca1p is responsible for the caspase activation signals detected in *orc2-1* cells with FITC-VAD-FMK. More cells with activated caspases were also detected in *orc2-1 yca1Δ*, but not wild-type cells transformed with an empty vector control plasmid in this experiment. This suggests the existence of additional, unidentified caspase-like molecules that are also activated by the *orc2-1* mutation. Deletion of *YCA1* also partly suppressed the temperature sensitivity associated with the *orc2-1* mutation (Fig. 2D). Therefore, Yca1p contributes to the lethal effects caused by loss of Orc2p function. This also contrasts with the results of (Wysocki and Kron, 2004), which failed to establish a role for Yca1p in the death of a different temperature-sensitive budding-yeast mutant.

Apoptosis is induced by loss of Orc2p function in G1 phase and subsequent entry into S phase

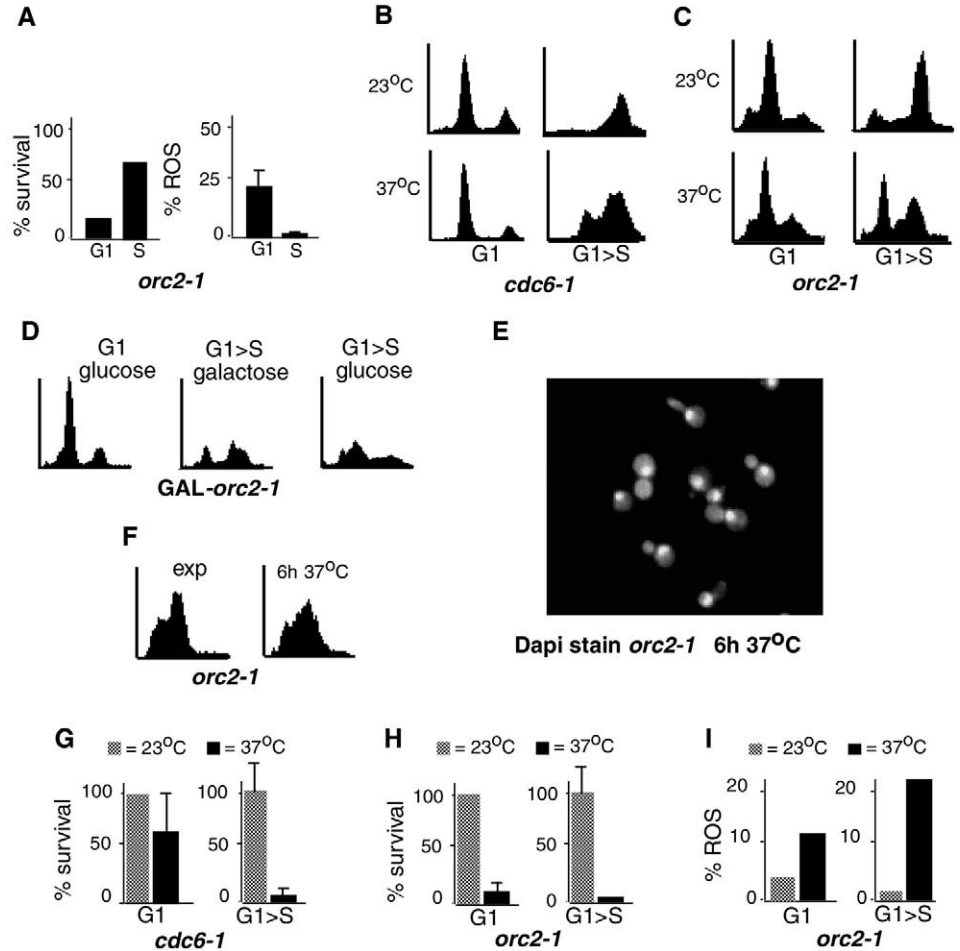
One function during G1 phase of ORC and other initiation proteins, including the ORC-interacting protein Cdc6, is to establish pre-RCs that 'license' origins of replication for activation in S phase (Diffley, 2001). As reported previously (Weinberger et al., 1999), loss of viability in *orc2-1* cells shifted to nonpermissive temperatures was significantly greater in cells first arrested in G1 phase with mating pheromone compared with cells synchronized in S phase before the temperature shift (Fig. 3A, left). More ROS-producing cells were also detected in G1-phase-arrested than in S-phase-arrested *orc2-1* cells (Fig. 3A, right). Thus, lethality and apoptosis in *orc2-1* cells are induced by loss of Orc2p function in G1 phase, and S-phase cells are more refractory to these effects.

Mating pheromone arrests cells in G1 after pre-RCs are assembled (Detweiler and Li, 1997). Therefore, although apoptosis associated with loss of Orc2p function in log-phase cultures of cells could be due to inhibition of the assembly of

Fig. 3. Cell-cycle-dependent effects on apoptosis in GA1410 *orc2-1* cells.

(A) Survival (left) and ROS production (right) in GA1410 *orc2-1* cells after synchronization in G1 or S phase for 3 hours at 23°C followed by a shift to 37°C for 3 hours. Viability measurements were normalized to viability of cells synchronized in G1 or S phase in the absence of the temperature shift to account for minor effects on viability of synchronization regimens. (B,C) FACS measurements of temperature-dependent effects on DNA replication in *cdc6-1* (B) and GA1410 *orc2-1* (C) cells synchronized in G1 with α mating factor and subsequently released into S phase. Cells were synchronized in G1 phase for 2 hours at 23°C and maintained in G1 phase for an additional 5 hours at this temperature or at 37°C ('G1'), or were synchronized in G1 phase for 2 hours at 23°C and then maintained at this temperature or at 37°C for an additional 3 hours followed by release into S phase for 2 hours at the same temperature ('G1>S').

(D) Effects of *orc2-1p* depletion on DNA replication in GAL-*orc2-1* cells that regulate *orc2-1* expression from a galactose-inducible and glucose-repressible promoter. (E) GA1410 *orc2-1* cells arrest in S phase after shifting to 37°C for 6 hours. DNA is stained with DAPI. (F) Effects of temperature on the cell cycle in exponentially proliferating GA1410 *orc2-1* cells. (G-I) Effects of temperature on viability of *cdc6-1* (G) and GA1410 *orc2-1* (H) cells, and on ROS production in GA1410 *orc2-1* cells (I) synchronized in G1 phase and then released ('G1>S') or not released ('G1') into S phase using the same regimen described for FACS analysis (B,C).



pre-RCs, apoptosis in pheromone-arrested cells might be caused by a failure to maintain pre-RCs at high temperatures during the pheromone-induced arrest, similar to the failure of Cdc6 to maintain pre-RCs in temperature-sensitive *cdc6-1* cells shifted to high temperatures during a pheromone arrest (Detweiler and Li, 1997; Piatti et al., 1995). Loss of this maintenance function in G1-arrested *cdc6-1* cells shifted to 37°C is probably responsible for the less-efficient DNA replication detected when these cells were subsequently released into S phase at this temperature, in contrast to the more-efficient DNA replication that occurred in *cdc6-1* cells synchronized in G1 and then released into S phase at the permissive temperature of 23°C (Fig. 3B). The *orc2-1* cells also replicated DNA less efficiently when shifted to 37°C after they were synchronized in G1 phase with pheromone and then released into S phase at 37°C, but not when they were synchronized and released from the pheromone block at 23°C (Fig. 3C). Less-efficient DNA replication in *orc2-1* cells was not related to combined effects of high temperature on *orc2-1* function and other processes, because GAL-*orc2-1* cells depleted of *orc2-1p* by glucose repression of transcription for 2 hours at 23°C instead of by high temperature also replicated DNA less efficiently when released from a mating-pheromone block in G1 phase (Fig. 3D). This contrasts with the lack of

inhibitory effects on DNA replication previously detected in these same cells after glucose inhibition of *orc2-1p* production for 30 minutes, which was interpreted as evidence that ORC is not required for maintenance of pre-RCs (Shimada et al., 2002). Our experiments used a 2-hour depletion of *orc2-1p* from GAL-*orc2-1* cells before release into S phase and, in our temperature shift experiments using GA1410 *orc2-1* cells, the temperature was raised for 3 hours before release occurred. An extended period of time required for disassembly of pre-RCs was previously observed in *cdc6-1* cells shifted to 37°C while blocked in G1 phase (Detweiler and Li, 1997) and in G1-phase cells depleted of the pre-RC protein Mcm2p for 1.5 hours by degenon-mediated degradation (Labib et al., 2001). Therefore, similar to Cdc6p and Mcm2p, Orc2p is required for maintenance of pre-RCs in budding yeast over longer periods of time. Consequently, apoptosis induced by high temperatures in *orc2-1* cells that were first synchronized in G1 phase with mating pheromone is probably caused by loss of this maintenance function.

In some genetic backgrounds, pre-RC and other initiation proteins are required for a G1/M-phase checkpoint that restrains mitosis in G1 cells (Toyn et al., 1995; Weinreich et al., 2001). Divided chromatin, which is a signature of a catastrophic mitosis, was sometimes detected in

unsynchronized cultures of *orc2-1* cells shifted to higher temperatures (not shown). However, in many experiments, apoptosis was detected in *orc2-1* cells that arrested with undivided chromatin (Fig. 3E) and a range of bud sizes indicating a premitotic arrest in S phase (Fig. 3E and Fig. 1A). Therefore, although G1/M-phase checkpoint defects and catastrophic mitosis could cause apoptosis in *orc2-1* cells, apoptosis appears to occur in S-phase cells as well.

In fact, significantly more S-phase cells were present in mating-pheromone-synchronized cultures of *orc2-1* cells than in *cdc6-1* cells, especially at 37°C (Fig. 3B,C, G1). Even this partial synchronization of *orc2-1* cells in G1 required much more mating pheromone than for *cdc6-1* or wild-type cells. This probably reflects partial derepression by the *orc2-1* mutation of ORC-dependent silencing in the *HML* mating-type locus, which is expected to cause a defect in the response of the *orc2-1* cells used in these experiments to α mating factor. Loss of viability in *orc2-1* cells shifted to nonpermissive temperatures has been shown to coincide with their entry into S phase (Bell et al., 1993). Therefore, apoptosis in populations of *orc2-1* cells synchronized in G1 phase might actually occur in a subset of cells that lost Orc2p function in G1 phase but then escaped the leaky G1 block by mating pheromone in these cells and entered S phase. Consistent with this possibility, shifting *cdc6-1* cells to higher temperatures while maintaining them in G1 phase caused less lethality than in *orc2-1* cells under similar conditions (Fig. 3G,H, G1). Moreover, both *cdc6-1* and *orc2-1* cells were significantly less viable when released into S phase from the G1-phase mating-pheromone arrest at 37°C (Fig. 3G,H, G1>S) than cells that were maintained in mating pheromone (Fig. 3G,H, G1). Therefore, loss of viability in both *cdc6-1* and *orc2-1* cells at higher temperatures requires loss of function in G1, but is exacerbated by subsequent entry into S phase. The increased lethality of *orc2-1* cells released into S phase was accompanied by an increase in the number of ROS-producing cells (Fig. 3I). Therefore, although apoptosis is induced in *orc2-1* cells by loss of Orc2p function in G1, it probably also requires subsequent entry into S phase.

Apoptosis in *orc2-1* cells is stimulated by Mec1p and coincides with activation of Rad53p

Apoptosis in metazoans often depends on proteins required for checkpoints (for a review, see Burhans et al., 2003). Checkpoints were previously implicated in the temperature sensitivity of the *orc2-1* mutation by partial suppression of this sensitivity at semipermissive temperatures in diploid *orc2-1* cells with *RAD9* deleted (Watanabe et al., 2002). The *mec1-21* mutation in the *MEC1* gene, whose product functions upstream of Rad9p in budding-yeast checkpoints, also partly suppresses the temperature sensitivity of the *orc2-1* mutation (Desany et al., 1998). Production of wild-type Mec1p stimulated ROS production at both semipermissive and nonpermissive temperatures in the same *orc2-1 mec1-21* double-mutant cells that showed partial suppression of *orc2-1* lethality (Desany et al., 1998) (Fig. 4A). Therefore, similar to temperature sensitivity, ROS production in *orc2-1* cells can partly depend on Mec1p.

The stimulation of ROS by ectopic production of Mec1p suggested that apoptosis in *orc2-1* cells might be caused by

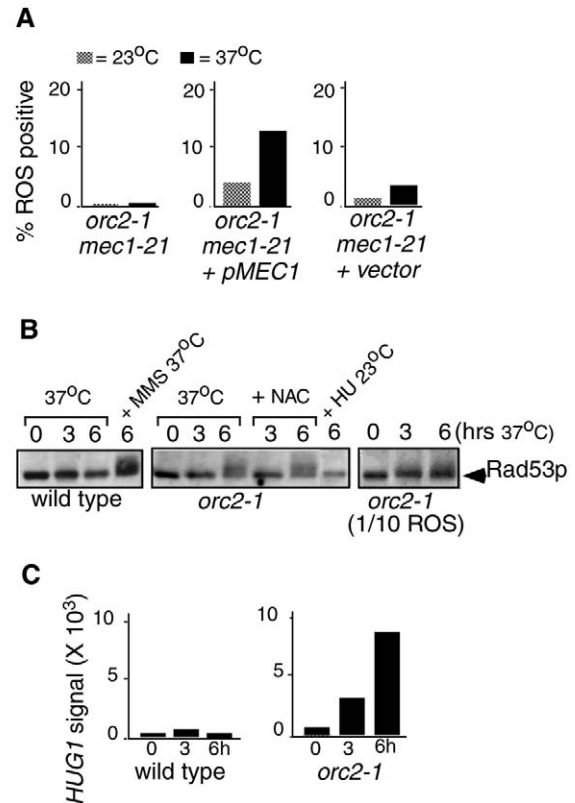


Fig. 4. Apoptosis in *orc2-1* cells is stimulated by the Mec1p checkpoint protein and coincides with a Rad53-dependent DNA-damage response. (A) Production of Mec1p stimulates the production of ROS in *mec1-21 orc2-1* cells. (B) Rad53p phosphorylation in GA1410 *orc2-1* and W303 wild-type cells. *orc2-1* (1/10 ROS) are strain YB0057. (C) Induction of *HUG1* expression in GA1410 *orc2-1* and wild-type cells. Signals are arbitrary units of fluorescently labeled cDNA produced from mRNA isolated from cells shifted to 37°C for indicated times and hybridized to *HUG1* sequences on an Affymetrix microarray chip. Values represent the average of two independent experiments without subtraction of background.

DNA damage. In wild-type cells, both DNA damage and stalling of DNA replication forks with the ribonucleotide reductase inhibitor hydroxyurea (HU) induce phosphorylation by Mec1p of the checkpoint kinase Rad53p. Rad53p was phosphorylated in *orc2-1* but not wild-type cells that had been shifted to 37°C (Fig. 4B). As expected, Rad53 phosphorylation was also observed in wild-type cells treated with the DNA-damaging agent methyl methanesulfonate (MMS) (Fig. 4B, +MMS 37°C). Therefore, loss of Orc2p function in *orc2-1* cells induces a Rad53p-dependent DNA-damage response, similar to the response induced in wild-type cells treated with MMS. Activation of Rad53p by DNA damage or HU in wild-type cells induces the expression of *HUG1* (Basrai et al., 1999). Rad53p phosphorylation induced by high temperatures in *orc2-1* cells coincided with an almost tenfold increase in expression of *HUG1* in *orc2-1* but not wild-type cells (Fig. 4C), confirming that Rad53p is activated upon loss of Orc2p function.

As reported earlier (Shimada et al., 2002), unlike wild-type cells, increased phosphorylation of Rad53p was not detected

in *orc2-1* cells treated with HU at 23°C (Fig. 4B, + HU 23°C), owing to their checkpoint defects. Therefore, phosphorylation of Rad53p and induction of *HUG1* expression at high temperatures in *orc2-1* cells cannot be a simple consequence of replication fork stalling in the absence of additional events, which probably include damage to DNA. Rad53p phosphorylation was also not a consequence of ROS-induced DNA damage, because it was not altered by the antioxidant *N*-acetyl cysteine (Fig. 4B, NAC), despite a five-times reduction in levels of ROS in *orc2-1* cells treated with this compound (data not shown). Furthermore, similar levels of Rad53p phosphorylation were induced by high temperatures in a second strain of *orc2-1* cells (YB0057) that produces ROS at one-tenth the frequency detected in the GA1410 *orc2-1* strain used in the experiments described above [Fig. 4B, *orc2-1* (1/10 ROS)]. These findings strongly suggest that loss of Orc2p function in *orc2-1* cells at high temperatures causes DNA damage independently of ROS (which can also damage DNA). Furthermore, because *orc2-1* cells are refractory to activation of Rad53p by low but not high levels of DNA damage (Shimada et al., 2002), loss of Orc2p function appears to induce extensive DNA damage, which could be responsible for the apoptotic phenotype of *orc2-1* cells at high temperatures.

Apoptosis is detected in other initiation mutants and is amplified by additional loci that respond to DNA damage

To determine whether apoptosis occurs in response to the inhibition of initiation complexes by other means, we asked whether it occurs in strains harboring mutations in other proteins required for the assembly of pre-RCs. Cells harboring the temperature-sensitive *cdc6-1* mutation also produced ROS when shifted to higher temperatures. However, the frequency with which ROS were produced in *cdc6-1* cells, although several hundred times higher than in wild-type cells, was much lower and occurred with delayed kinetics compared with the GA1410 *orc2-1* strain (Fig. 5A). ROS were also detected at levels significantly higher than in wild-type cells in several additional *orc2-1* strains shifted to 37°C or depleted of *orc2-1p* by glucose repression (Table 1), as well as in *orc2-1* strains in other genetic backgrounds shifted to 37°C (data not shown). However, like *cdc6-1* cells, in all of these strains, the frequency of ROS production was lower than in GA1410 *orc2-1* cells. The lower frequency of ROS production in *cdc6-1* and other *orc2-1* strains was not related to the potential accumulation of mutations in these strains that suppressed production of ROS, because ROS levels did not increase after extensive backcrossing of these strains with wild-type cells to remove suppressors (data not shown). By contrast, although most *orc2-1* segregants of a backcross between GA1410 *orc2-1* and wild-type cells produced ROS at high temperatures at a frequency similar to GA1410 *orc2-1* cells, some segregants of this backcross produced ROS at a frequency more similar to *cdc6-1* and several other *orc2-1* strains (Fig. 5B, *orc2-1* segregants). In addition, at high temperatures, many of the wild-type *ORC2*

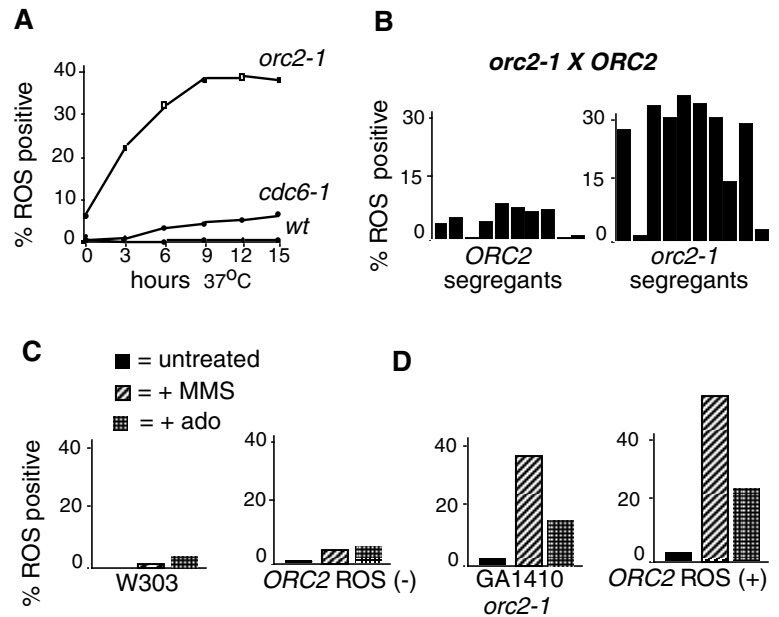


Fig. 5. ROS is induced in other temperature-sensitive initiation mutants and is amplified by additional loci that also respond to DNA damage.

(A) Temperature- and time-dependent induction of ROS in *cdc6-1*, GA1410 *orc2-1* and wild-type (W303) cells. (B) ROS induction in wild-type and *orc2-1* segregants of a GA1410 *orc2-1* × *ORC2* mating shifted to 37°C for 6 hours. (C) ROS induction by DNA-damaging agents in parental wild-type (W303) or GA1410 *orc2-1* cells. (D) ROS induction by DNA-damaging agents in wild-type (*ORC2*) segregants of the GA1410 *orc2-1* × *ORC2* mating that do [ROS (+)] or do not [ROS (-)] harbor the additional determinants of ROS present in GA1410 *orc2-1* cells. Cells were exposed for 6 hours at 23°C to the DNA-damaging agent adozelesin (ado) at a final concentration of 4 μM or to 0.1% methylmethane sulfonate (MMS).

Table 1. ROS production in wild type and mutant *ORC2* strains

Strain	Genotype	Stimulus (6 hours)	ROS-positive (in %)
W303	wild type	37°C	<0.1
GA1410	<i>orc2-1</i> (MATa)	37°C	32.0
GA1411	<i>orc2-1</i> (MATα)	37°C	3.0
YB0057	<i>orc2-1</i>	37°C	3.0
GA1680	GAL- <i>orc2-1</i>	galactose	<0.1
GA1680	GAL- <i>orc2-1</i>	glucose	7.4
YB0059	<i>orc5-1</i>	37°C	<0.1
YMW101	<i>ORC2</i> ROS ⁺	37°C	7.0
YMW102	<i>orc2-1</i> (YB0057) × <i>ORC2</i> ROS ⁺	37°C	34.6
YMW103	<i>orc5-1</i> (YB0059) × <i>ORC2</i> ROS ⁺	37°C	21.5

ROS measurements were made after subjecting cells to the indicated stimulus. All cells are in the W303 background.

segregants of this backcross produced ROS at a low frequency, although this was still significantly higher than the very low frequency (<0.1%) detected in parental wild-type cells (Fig. 5B, *ORC2* segregants). These findings confirmed that high levels of ROS in GA1410 *orc2-1* cells depend on the *orc2-1* mutation but suggested that GA1410 *orc2-1* cells harbor additional loci that stimulate ROS and segregate independently of the *orc2-1* mutation. This was confirmed by measuring ROS

Table 2. Strains and plasmids

	Genotype	Source
Strain		
W303-1A	<i>MATa, ade2-1, ura3-1, his3-11, tro1-1, leu2-3, 112, can1-100</i>	S. Gasser
GA1410	W303 <i>MATa, orc2-1</i>	S. Gasser
GA1411	W303 <i>MATa, orc2-1</i>	S. Gasser
GA1680	W303 <i>MATa, GAL-orc2-1</i>	S. Gasser
YB0044	W303 <i>MATa, cdc6-1</i>	B. Stillman
YB0057	W303 <i>MATa, orc2-1</i>	B. Stillman
YB0059	W303 <i>MATa, orc5-1</i>	B. Stillman
Y612	W303 <i>MATa, orc2-1, mec1-21</i>	S. Elledge
MWY101	<i>MATa, ORC2 ROS⁺</i> (From GA1410 × W303)	This study
MWY 102	<i>MATa, orc2-1 ROS⁺</i> (From YB0057 × <i>ORC2 ROS⁺</i>)	This study
MWY103	<i>MATa, orc5-1 ROS⁺</i> (From YB0059 × <i>ORC2 ROS⁺</i>)	This study
MWY143	GA1410, <i>yca1Δ</i>	This study
Plasmids		
pFM21	<i>YCA1</i> cloned in pESC-HIS	F. Madeo
pMEC	<i>GAL-MEC1-HA</i>	G. Brush

levels in segregants of a cross between YB0057 *orc2-1* cells (which produce ROS at a frequency of 3%) and wild-type *ORC2* segregants [*ORC2* (ROS⁺)] from the GA1410 *orc2-1* × *ORC2* mating (which produce ROS independently of the *orc2-1* mutation at a frequency of 7%). ROS were detected in *orc2-1*-containing segregants of this backcross at a much higher frequency (~35%) than either parent and at a frequency very similar to that detected in GA1410 *orc2-1* cells (Table 1, YMW102). Similarly, although ROS production was not detected above background wild-type levels in a different *ORC2* mutant, *orc5-1* (Table 1, YB0059), some *orc5-1*-containing segregants of a cross between *orc5-1* and *ORC2* (ROS⁺) cells produced ROS at high frequencies, similar to the frequency of ROS production in GA1410 *orc2-1* cells (Table 1, YMW103). The detection of ROS in other *orc2-1* strains and in association with mutations in other initiation proteins indicates that apoptosis is a general consequence of defects in the assembly and maintenance of pre-RCs. However, in GA1410 *orc2-1* cells, ROS levels are increased by additional temperature-sensitive loci residing in this strain. At high temperatures, these loci produce ROS independently of the *orc2-1* mutation but at much lower levels than detected in GA1410 *orc2-1* cells.

The amplification of ROS by undefined loci in GA1410 *orc2-1* cells in concert with the induction of a Rad53p-mediated DNA damage response suggested that these loci respond to DNA damage. Consistent with earlier reports that ultraviolet radiation (Del Carratore et al., 2002) or the *cdc13-1* mutation – which causes the accumulation of DNA damage (Qi et al., 2003) – induce apoptosis in budding-yeast cells, MMS or adozelesin (an experimental antitumor drug that, like MMS, damages DNA) induced ROS in W303 wild-type cells, albeit at a low frequency compared with GA1410 *orc2-1* cells (Fig. 5C, W303). ROS were also induced at a low frequency in a wild-type *ORC2* segregant of the GA1410 *orc2-1* × *ORC2* backcross that produces very little ROS at high temperatures, and therefore lacks the additional ROS-amplifying loci [*ORC2* ROS (–)]. By contrast, exposure to adozelesin or MMS at 23°C induced ROS at a much higher frequency in GA1410 *orc2-1* cells (Fig. 5C, GA1410 *orc2-1*) and in a wild-type *ORC2* segregant of the GA1410 *orc2-1* × W303 backcross, both of

which contained these loci {Fig. 5C, *ORC2* [ROS (+)]}. In fact, despite the performance of this experiment at a lower temperature that produces much less ROS in GA1410 cells, the frequency of ROS induced in these cells by DNA-damaging agents equaled or exceeded the frequency of ROS production in GA1410 *orc2-1* cells at high temperatures. Therefore, the additional loci in GA1410 *orc2-1* cells that amplify the frequency of ROS induced at high temperatures also enhance the apoptotic response of budding-yeast cells to DNA damage independently of the *orc2-1* mutation.

Discussion

DNA damage and apoptosis in budding-yeast initiation mutants

Important but poorly understood connections exist between cell-cycle-regulatory pathways and apoptosis in mammalian cells. In addition to the inappropriate activation of S phase and mitotic CDKs (Castedo et al., 2002; Guo and Hay, 1999; King and Cidlowski, 1995; Lukovic et al., 2003; Meikrantz and Schlegel, 1995), which can be required for apoptosis (for reviews, see Castedo et al., 2002; Gartel and Tyner, 2002; Guo and Hay, 1999), apoptosis is often accompanied by the caspase- and/or proteasome-dependent destruction of multiple DNA replication and/or checkpoint proteins that directly or indirectly inhibit CDK activity (Blanchard et al., 2002; Flygare et al., 2000; Huang et al., 1999; Itoh and Horio, 2001; Lee et al., 2003; Levkau et al., 1998; Pelizon et al., 2002; Smith et al., 1999; Tan et al., 1997; Yim et al., 2003; Zhou et al., 1998). Apoptosis also coincides with the caspase destruction of proteins required for DNA repair (Flygare et al., 2000; Huang et al., 1999; Itoh and Horio, 2001). Caspase-dependent destruction of many of these DNA replication, checkpoint and DNA repair proteins is required for full induction of the apoptotic machinery, because non-caspase-cleavable mutants of some of these proteins can block apoptosis (Gartel and Tyner, 2002; Huang et al., 1999; Lee et al., 2003; Pelizon et al., 2002; Tan et al., 1997; Yim et al., 2003). Therefore, despite the frequent assumption that the commitment to cell death during apoptosis must occur upon activation of caspases, in at least some cases this commitment clearly occurs downstream of the activation of caspases and requires the simultaneous inhibition of DNA replication and checkpoints in concert with the unscheduled activation of CDKs and the induction of DNA damage. The nature of this commitment is not clear.

The findings reported here establish that, in budding yeast, mutations that partly uncouple DNA replication from the cell cycle by simultaneously reducing the number of pre-RCs and attenuating checkpoints induce DNA-damage responses and an apoptosis-like phenotype. This phenotype includes the production of ROS (Fig. 1) and activation of Yca1p, a recently described budding-yeast metacaspase (Fig. 2). A role for Yca1p in yeast apoptosis and the validity of the concept of apoptosis in yeasts was recently challenged by experiments that used extraordinarily lethal conditions allowing for a maximum of 0.001% survival (Wysocki and Kron, 2004). Unlike the Wysocki and Kron study, but similar to other studies that (like our study) used far less lethal conditions more appropriate for studying cell-death pathways (Bettiga et al., 2005; Fahrenkrog et al., 2004; Madeo et al., 2002b; Wadskog et al., 2004) (C. Mazzoni et al., personal communication), our

experiments in *orc2-1* cells clearly show that disruption of *YCA1* in budding-yeast cells undergoing apoptosis-like cell death substantially reduces caspase activation signals in parallel with a significant increase in viability (Fig. 2). In conjunction with the production of ROS (Fig. 1) and an earlier report of DNA degradation and checkpoint-dependent cell death induced by the *orc2-1* mutation (Watanabe et al., 2002), these results establish that many of the *orc2-1* cells used in our experiments were undergoing an apoptosis-related cell death.

Apoptosis in *orc2-1* cells requires loss of Orc2p function in G1 and subsequent entry into S phase or mitosis (Fig. 3). Although loss of Orc2p function is expected to reduce the number of pre-RCs in G1 cells by inhibiting pre-RC formation, our results indicate that, in budding yeast, Orc2p is also required for maintaining pre-RCs once they are formed. Although this conclusion is contrary to that of Shimada et al. based on a similar experiment (Shimada et al., 2002), depletion of *orc2-1p* in that earlier study might not have occurred for long enough to allow the disassembly of pre-RCs.

Apoptosis in *orc2-1* cells is accompanied by activation of the checkpoint protein Rad53p (Fig. 4B). Owing to the attenuation of checkpoints by a reduction in the number of replication forks below a threshold required for full checkpoint activation, *orc2-1* cells are refractory to activation of Rad53p by stalled replication forks or low levels of DNA damage (Fig. 4) (Shimada et al., 2002). Therefore, Rad53p activation at high temperatures in *orc2-1* cells is probably induced by extensive DNA damage associated with acute loss of ORC function. In metazoans, apoptosis is induced by DNA damage that exceeds the capacity for repair. We consider it likely that extensive DNA damage exceeding capacity for repair is also responsible for the apoptotic phenotype of initiation mutants in budding yeast.

DNA damage caused by initiation defects in our experiments is consistent with recent evidence that, in yeast and mammals, defects in initiation activate DNA-damage responses (Jacobson et al., 2001; Kim et al., 2002; Liang et al., 1999; Watanabe et al., 2002) and cause gross chromosomal rearrangements (GCRs) (Huang and Koshland, 2003; Lengronne and Schwob, 2002; Tercero et al., 2003; Watanabe et al., 2002). Why defects in initiation cause these effects has not been clear. DNA damage could be caused by inappropriate entry of cells into anaphase with partly replicated chromosomes, as has been detected in budding-yeast cells with fewer pre-RCs owing to unscheduled activation of S-phase CDKs (Lengronne and Schwob, 2002). It was proposed that the absence of checkpoint responses observed in these experiments reflected the failure of budding-yeast cells to evolve mechanisms for sensing ongoing DNA replication when an extended period of time is required to finish replicating chromosomes in cells with reduced numbers of origins (Lengronne and Schwob, 2002). Genome instability associated with defects in initiation has also been suggested to arise during S phase in the absence of DNA damage, owing to elevated recombination at intrinsically stalled replication forks that require more time for rescue by forks emanating from more distal origins (Tanaka and Diffley, 2002).

Both these models fail to consider the dual role of initiation complexes in DNA replication and in checkpoints that directly or indirectly depend on initiation complexes (Shirahige et al., 1998a; Tercero et al., 2003; Weinberger et al., 1999). In this

context, DNA damage associated with inappropriate entry of initiation mutants into mitosis with incompletely replicated chromosomes is more likely to be caused by checkpoint failure than by a failure to evolve checkpoints in cells with an extended S phase. Similarly, DNA damage caused by checkpoint failure, rather than recombination in the absence of DNA damage, might underlie genome instability that arises during S phase in strains with partial defects in initiation. Checkpoints stabilize stalled DNA replication forks, which collapse in checkpoint-defective cells (Sogo et al., 2002). Induction of DNA damage during S phase, as well as mitosis, is consistent with the Rad9-dependent G2 arrest detected in these strains, including those harboring the *orc2-1* mutation (Jacobson et al., 2001; Watanabe et al., 2002).

Apoptosis in initiation mutants as a model for apoptosis in cycling mammalian cells

Our findings suggest a model for how the targeted destruction of highly conserved DNA replication initiation, checkpoint and repair proteins, and activation of CDKs might contribute to apoptosis in mammalian cells. Similar to budding yeast, this probably leads to DNA damage when cells with a reduced number of origins and defective checkpoint and DNA repair mechanisms enter mitosis with incompletely replicated DNA. This is consistent with an extensive literature describing the induction by a variety of stimuli of an apoptosis-like cell death that includes mitotic catastrophe driven by activation of the mitotic CDK, CDK1 (Castedo et al., 2002). The relationship between apoptosis and mitosis is not entirely clear, however, partly because some mitotic events are not always detected in apoptotic cells (Zhou et al., 1998). The detection of apoptosis in *orc2-1* cells in S phase (Fig. 1A) in the absence of a mitotic catastrophe (Fig. 3E) suggests an additional possibility – that DNA damage leading to apoptosis can be induced by uncoupling DNA replication from S-phase, as well as mitotic, events. This is consistent with the activation during apoptosis of CDK2/cyclin-A, which functions in S phase and is required for apoptosis in some cases (Meikrantz et al., 1994). Subsequent full engagement of the apoptotic machinery in S phase might lead to abortive mitotic entry or even block mitosis altogether, depending on other factors, such as the status of parallel p53-dependent mitotic checkpoints.

As in budding yeast, simultaneous inhibition of DNA replication and checkpoints during apoptosis is expected to cause DNA damage specifically at replication forks that collapse while stalled in S phase or during mis-segregation of partly replicated chromosomes during mitosis. Interestingly, several studies indicate that, during early stages of apoptosis induced by a range of apoptotic stimuli in mammalian cells, large fragments of DNA (50 kb to 1 Mb) corresponding to looped domains of chromatin are detached from chromosomes before detection of the nucleosome-sized fragments of DNA at later stages of apoptosis (Li et al., 1999; Ploski and Aplan, 2001; Solovyan et al., 2002) (for reviews, see Higuchi, 2003; Nagata et al., 2003). The production of these large replication-sized DNA fragments can amplify an initial low caspase-dependent apoptotic response, thus establishing a causal role for these fragments in the apoptotic program downstream of the activation of caspases (Boulares et al., 2001). Although the mechanism by which these DNA fragments are produced

remains unclear, topoisomerases I and II (both of which are essential for DNA synthesis at replication forks) have been implicated in their formation during the execution phase of apoptosis (for a review, see Sordet et al., 2003). Other experiments suggest that stalled replication forks can serve as 'suicide' substrates for topoisomerases (Snapka et al., 1991). In the context of our findings and the generally accepted notion that DNA replication takes places at the base of looped domains of chromatin, there is the interesting possibility that some of the large, replicon-sized fragments detected at early stages of apoptosis are produced by topoisomerase-mediated replication-fork collapse when DNA replication becomes uncoupled from subsequent cell-cycle events. This model clearly does not extend to all forms of apoptosis. We think it is possible, however, that it describes a conserved core apoptotic program that might have been augmented or superseded by the evolution of more specialized mechanisms in differentiated cells of multicellular organisms.

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