Saccharomyces cerevisiae Dap1p, a Novel DNA Damage Response Protein Related to the Mammalian Membrane-Associated Progesterone Receptor

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The response to damage is crucial for cellular survival, and eukaryotic cells require a broad array of proteins for an intact damage response. We have found that the YPL170W (DAP1 [for damage response protein related to membrane-associated progesterone receptors]) gene is required for growth in the presence of the methylating agent methyl methanesulfonate (MMS). The DAP1 open reading frame shares homology with a broadly conserved family of membrane-associated progesterone receptors (MAPRs). Deletion of DAP1 leads to sensitivity to MMS, elongated telomeres, loss of mitochondrial function, and partial arrest in sterol synthesis. Sensitivity of dap1 strains to MMS is not due to loss of damage checkpoints. Instead, dap1 cells are arrested as unbudded cells after MMS treatment, suggesting that Dap1p is required for cell cycle progression following damage. Dap1p also directs resistance to itraconazole and fluconazole, inhibitors of sterol synthesis. We have found that dap1 cells have slightly decreased levels of ergosterol but increased levels of the ergosterol intermediates squalene and lanosterol, indicating that dap1 cells have a partial defect in sterol synthesis. This is the first evidence linking a MAPR family member to sterol regulation or the response to damage, and these functions are probably conserved in a variety of eukaryotes.

Eukaryotic cells are constantly exposed to exogenous and endogenous damage. Cells respond to DNA damage by delaying cell cycle progression (27), repairing the damage, and activating or repressing transcription of a number of genes (19, 28). Mutants that are unable to activate the checkpoint pathway continue to replicate damaged DNA and die (56). Checkpoint proteins include the protein kinase Mec1p (1, 34, 40, 57) and Rad9p (56). Some yeast damage repair mutants have a functioning checkpoint response and are capable of delaying the cell cycle but cannot repair the damaged lesion. In general, yeast DNA repair mutants fall into three categories: mutants affected in nucleotide excision repair, postreplication repair, and recombinational repair (18).

Yeast requires a large number of processes to respond to damage. Diploid yeast contains 130 nonessential genes that are required for the response to ionizing radiation, and 100 of these genes are required for the response to methyl methanesulfonate (MMS) (3). The total number of genes required for the response to MMS is not known, and it is not clear which of the radiation-sensitive diploids are sensitive as haploids. In addition to proteins regulating the damage checkpoint and recombinational repair, diploid yeasts require proteins directing replication, chromatin silencing, and mitotic chromosome transmission for the response to MMS (3). Genes with less direct roles in DNA metabolism are also required for the response to MMS, including genes encoding the nuclear pore complex; vacuolar, Golgi, and endocytosis components; and members of the ubiquitin degradation pathway and genes regulating transcription and RNA metabolism (3). Finally, deletion of the ERG3 gene, encoding C-5 sterol desaturase, leads to MMS sensitivity, and erg28 (17) and arv1 (for ARE2 required for viability) (53) strains are sensitive to a lesser extent (3). Thus, the response to ionizing radiation and MMS is complex and requires the function of a number of genes, including some genes that function in sterol synthesis.

In yeast, several proteins that regulate the DNA damage response are also required for telomere length maintenance (reviewed in reference 4). Damage repair proteins probably serve protective functions for telomeres, distinguishing them from a double-stranded DNA break. Ku70p localizes to telomeres in the absence of damage and then translocates to sites of damage following double-stranded DNA breaks (35, 36, 38). The telomere binding proteins Sir3p, Sir4p, and Rap1p also diffuse from the telomere after damage. Following translocation from the telomere, these proteins probably serve to cap broken DNA ends and block replication and transcription in the DNA adjacent to the break, facilitating the processing and repair of these regions. A large number of proteins that contribute to checkpoint or damage repair also regulate telomere length, including members of the Mre11p-Rad50p-Xrs2p complex (5), Mec1p (43), Mec3p (6), Rad27p (41), and Tel1p (21, 33).

We have searched for novel yeast genes that regulate the damage response. The murine 25-Dx protein is induced following exposure of the carcinogen 2,3,7,8-tetrachloro-p-dioxin, suggesting a role in the response to cellular stress and in carcinogenesis (46). 25-Dx is part of a highly conserved family of proteins that is collectively called the membrane-associated progesterone receptor (MAPR) family. The porcine MAPR
binds to progesterone and was isolated from liver membrane fractions (14, 37). However, the biological function of MAPRs in progesterone signaling is unknown.

The yeast open reading frame YPL170W encodes a protein that is part of the MAPR family (Fig. 1). The predicted protein product from the YPL170W open reading frame shares homology throughout its coding sequence with its human and rodent counterparts (Fig. 1A) and has a region identical to proteins from a broad variety of organisms, including Arabidopsis thaliana and Schizosaccharomyces pombe (Fig. 1B). Many of these proteins are similar in molecular weight and contain the homologous region near the center of the coding sequence (Fig. 1C).

Because of the role of 25-Dx in carcinogenesis, we deleted the YPL170W open reading frame and have characterized the phenotypes of the mutant strain. We have found that the phenotypes of the YPL170WΔ strain are pleiotropic, affecting growth in response to damage, telomere length, and sterol regulation. Our results are the first genetic analysis of a MAPR family member and indicate new roles for these proteins in the damage response and cellular metabolism.

MATERIALS AND METHODS

Yeast strains and growth conditions. All strains were isogenic with W303 (leu2-3, 112 his3-11, 15 can1-100 rad5-1 ade2-1 trp1-1 can1-100 rad5-355) (52) except for the alterations described in Table 1. In most of the strains, the rad5-355 allele was replaced with the wild-type RAD5 gene by crossing and tested by PCR as described previously (9). In some strains, the type X subtelomeric repeat element on the left telomere of chromosome XV was replaced with URA3 (designated XVL-TEL-URA3) as described previously (8).

The DAP1 gene was replaced with the LEU2 gene by a one-step transplace-
ment. The LEU2 gene was amplified from plasmid pRS305 (49) by using the primers DAPI-KOF and DAPI-KOR, so that the PCR product contained the entire LEU2 gene with flanking homology to DAPI. Strains were grown at 30°C in yeast extract-peptone-dextrose (YPD) or synthetic media (22). All primer sequences are available on request.

All genetic manipulations were performed essentially as described previously (22). The haploid strains used in these studies were primarily derivatives of diploid strains. The diploids were constructed by crossing the indicated strains (22). The haploid strains used in these studies were primarily derivatives of W303 strains. Log-phase cells were centrifuged, washed once in distilled water, and resuspended in a starting solution and then dividing by the absorbance of the starting solution. The percent crystal violet uptake was calculated by using the method of the median (32). Independent cultures were analyzed in two separate assays. The rate of canavanine resistance was calculated by using the method of the median (32).

Membrane permeability assays were performed essentially as described previously (2). Yeast cultures were grown to a density of 5 × 10⁶ cells/ml and incubated at various temperatures for 10 min and centrifuged, and the dye concentration in the supernatant was measured by optical density (OD) at 595 nm. The percent crystal violet uptake was calculated by subtracting the absorbance of cell supernatants from the absorbance of the starting solution and then dividing by the absorbance of the starting solution.

To assay resistance to a cation pulse, yeast cultures were grown to an OD of 1, and 1 ml of cells was removed and centrifuged. Cell pellets were mixed with 50 μl of a 2 M solution of CaCl₂, CrCl₃, KCl, MgCl₂, or NiCl₂ (all were purchased from Sigma) and incubated at 30°C for 10 min. Cells were then centrifuged at 4°C. For each sample, 25 μl was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in a 15% acrylamide gel, transferred to HyBond C (Amersham), and probed with an antibody to the Myc (9B11; Cell Signaling) epitope tag sequence.

**Southern blot analysis.** Genomic DNA was isolated from 5-ml cultures grown in YPD or selective media overnight at 30°C. DNA purification was performed as previously described (22). DNA was digested with PstI, separated by 1% agarose gel electrophoresis, and transferred to HyBond N membranes (Amer- sham). Blots were probed with a DNA fragment of the Y subtelomeric repeat, amplified from plasmid pYT14 by PCR with the primers TELO-5 and TELO-3, as described previously (7).

**Assay for mitochondrial maintenance, canavanine resistance, membrane permeability, and cation pulse resistance.** For assays of mitochondrial maintenance, cells were grown on YPD plates for 2 to 3 days. Colonies were picked, suspended in 0.5 ml of water, and diluted 10⁴, and 100 μl was plated on YPD plates. The plates were incubated at 30°C for 2 days and then left at 4°C for 3 to 7 days, and the numbers of red and white colonies were counted. Colonies were verified as petite by patching at least 100 colonies on plates containing 2% glycerol as a carbon source. For each assay, at least 15 colonies were plated, and the percentage of petite colonies was calculated for each plate and averaged.

Canavanine resistance assays were performed as previously described (10). Briefly, overnight cultures were diluted in water and plated onto plates lacking arginine, with or without 60 mg of l-canavanine (Sigma) per liter. Twenty independent cultures were analyzed in two separate assays. The rate of canavanine resistance was calculated by using the method of the median (32).

**Drug sensitivity assays and cell cycle analysis.** Overnight cultures were serially diluted 1:10 in water. Five microliters of the diluted cells was then spotted onto plates containing 20 mM hydroxyurea (Sigma) or 0.01% MMS (Sigma). For the overn, the plates were incubated to the same temperature as the culture) was added to a plate. After 3 days, the plates were stained with crystal violet and the numbers of colonies were counted. Canavanine resistance assays were performed as previously described (10). Briefly, overnight cultures were diluted in water and plated onto plates lacking arginine, with or without 60 mg of l-canavanine (Sigma) per liter. Twenty independent cultures were analyzed in two separate assays. The rate of canavanine resistance was calculated by using the method of the median (32).

Membrane permeability assays were performed essentially as described previously (2). Yeast cultures were grown to a density of 5 × 10⁶ cells/ml and incubated at various temperatures for 5 min, and crystal violet (Sigma) (preincubated to the same temperature as the culture) was added to a final concentration of 5 μg/ml. Cells were then incubated at various temperatures for 10 min and centrifuged, and the dye concentration in the supernatant was measured by optical density (OD) at 595 nm. The percent crystal violet uptake was calculated by subtracting the absorbance of cell supernatants from the absorbance of the starting solution and then dividing by the absorbance of the starting solution.

| Strain        | Relevant genotype                      | Reference |
|---------------|----------------------------------------|-----------|
| W303          | ade2-1 can1-100 his3-11,15 leu2-3,112 rad5-535 trpl-1 ura3-1 | 52        |
| HLK1042-1C    | α CAN1 hom5-10 RAD5                     |           |
| JMY37-1d      | α RAD53-13myc (::KanMX)                 |           |
| RCY23         | tel1::ura3 V₅ₐ₃::TEL-URA3               | 8         |
| RCY61-1b      | α nrm3 Δ::KanMX nrm2Δ::HIS3 tel1::ura3  |           |
| RCY90         | dap1Δ::LEU2 V₅ₐ₃::TEL-URA3              |           |
| RCY93-2a      | dap1Δ::LEU2 tel1::ura3                 |           |
| RCY93-4c      | α dap1Δ::LEU2                          |           |
| RCY93-7c      | Wild type a                            |           |
| RCY269-3d     | mec1-21 RAD5 XV₅ₐ₃::TEL-URA3           | 9         |
| RCY278-1a     | α mec1-21 RAD5 tel1::KanMX XV₅ₐ₃::TEL-URA3 | 9       |
| RCY300-6a     | α dun1-100::HIS3 RAD5 XV₅ₐ₃::TEL-URA3   | 9         |
| RCY307-4a     | CAN1 RAD5 rad9Δ::KanMX                 | 10        |
| RCY327-3c     | hom3-10 RAD5 rad9Δ::KanMX              | 10        |
| RCY344-2b     | CAN1 dap1Δ::LEU2 RAD5 V₅ₐ₃::TEL-URA3   |           |
| RCY406-1d     | RAD5 rad9Δ::KanMX                      |           |
| RCY406-5a     | dap1Δ::LEU2 RAD5 rad9Δ::KanMX          |           |
| RCY407-1d     | CAN1 dap1Δ::LEU2 RAD5                  |           |
| RCY407-5a     | α CAN1 dap1Δ::LEU2 dun1Δ::HIS3 RAD5 XV₅ₐ₃::TEL-URA3 | 9       |
| RCY407-12d    | RAD5                                   |           |
| RCY409-2a     | Wild type a                            |           |
| RCY409-4b     | α CAN1 dap1Δ::LEU2 RAD5                |           |
| RCY409-5c     | α CAN1 dun1Δ::HIS3 RAD5 XV₅ₐ₃::TEL-URA3 |           |
| RCY429-1a     | α RAD5                                 |           |
| RCY429-1d     | dap1Δ::LEU2 RAD5 RAD53-13myc (::KanMX) |           |
| RCY429-2b     | α RAD5 RAD53-13myc (::KanMX)           |           |
| SPY40         | tel1::URA3                             | 42        |
centrifuged, resuspended in phosphate-buffered saline, and plated for viability on YPD plates. All assays were performed in triplicate and duplicated in a separate analysis. 

Statistical analysis. To determine the percentage of total cellular mass represented by sterol, quantitative sterol analysis was performed as described by Molzahn and Woods (39). Briefly, cells were grown in YPD to an OD at 660 nm of 0.8 to 0.9. Cells were pelleted, washed once with dH2O, and then resuspended in 10 ml of alcoholic KOH (4.5 M KOH in 60% ethanol) and transferred to a clean round-bottom flask. The suspension was allowed to reflux at 88 to 90°C for 60 min. One milliliter of 95% ethanol was added down the condenser, refluxing was continued for another 60 min, and then the apparatus was cooled to room temperature. The condenser was removed, and 4 ml of dH2O and 10 ml of n-heptane were added to the flask before shaking vigorously for 2.5 min.

Two milliliters of the n-heptane layer (containing sterol) was transferred to a clean vial, and 3 μl of sample was analyzed by gas chromatography as described below. To calculate the percentage of sterols per milligram of cell mass, 20 ml of the original culture was harvested by vacuum filtration onto a preweighed 0.45-μm-pore-size Millipore filter. The filters were predried in a 105°C oven overnight and subsequently placed in a dessicator for 4 h. After vacuum filtration of harvested cells, the heating-desiccation step was repeated, and the weight of the cells was determined. The amount of individual sterols was calculated based on the area under each peak of the chromatograph relative to a known amount of ergosterol loaded onto the gas chromatograph, using the Hewlett-Packard sterol quantitation program. Each sample was injected twice, and the value reported for each sterol quantity was the average of those for two injections.

Sterols were analyzed by gas chromatography with a Hewlett-Packard HP5890 series II chromatograph equipped with the Hewlett-Packard CHEMSTATION software package. The capillary column (DB-1; J&W Scientific) was programmed from 195 to 280°C (1 min at 195°C and then an increase at 20°C/min to 240°C, followed by an increase at 2°C/min until the final temperature of 280°C was reached). The linear velocity was 30 cm/s, nitrogen was the carrier gas, and all injections were run in the splitless mode.

RESULTS

Identification of DAPI. YPL170W is a 456-bp open reading frame encoding a 152-amino-acid protein with a predicted molecular mass of 16.7 kDa. There is little homology between the YPL170W open reading frame and other yeast open reading frames, except for a 52-amino-acid region with 39% identity with the anaphase checkpoint protein Pds1p (not shown). However, YPL170W contains strong homology with a group of proteins that is collectively called the MAPR family (Fig. 1A). The rat and human homologues of YPL170W resemble the YPL170W open reading frame in size and contain homology to YPL170W throughout their coding sequences. The MAPR family includes 25-Dx (37, 46), the human 25-Dx homologues Hpr6.6 and Dg6 (20) (Fig. 1B), and uncharacterized family members in S. pombe, A. thaliana (Fig. 1B), and Caenorhabditis elegans.

We deleted the entire YPL170W open reading frame by one-step transplacement with the LEU2 gene. The following sections describe the phenotypes associated with loss of YPL170W function: damage sensitivity, telomere elongation, partial loss of ergosterol synthesis, and defects in mitochondrial biogenesis. Because YPL170WΔ mutants are damage sensitive and YPL170W contains homology to the MAPR family, we propose the name DAPI (for damage response protein related to the membrane-associated progesterone receptor) for YPL170W.

Cells lacking DAPI are sensitive to MMS. As part of our characterization of dap1Δ mutants, we tested the ability of dap1Δ strains to grow in the presence of damaging agents. We found that dap1Δ strains were unable to grow on plates containing 0.01% MMS (Fig. 2, middle panel, second row). The sensitivity of dap1Δ strains to MMS was completely reversed by a single-copy plasmid containing the entire DAPI open reading frame.

The dap1Δ strains were only mildly sensitive to the ribonucleotide reductase inhibitor hydroxyurea, with reduced growth at 20 mM (Fig. 2, lower panel, second row). This dose of hydroxyurea was toxic to cells lacking the Dun1p damage repair protein (Fig. 2, lower panel, bottom row). UV light and gamma irradiation had no effect on growth of haploid dap1Δ strains, even at doses that killed checkpoint mutant strains. However, diploid dap1Δ strains were approximately 10-fold more sensitive to ionizing and UV radiation than a comparable diploid wild-type strain, suggesting that Dap1p contributes to the growth response to radiation in diploid cells.

We surmised that MMS sensitivity in dap1Δ strains might be due to a checkpoint defect. Yeast cells arrested in G1 delay budding when damaged (47), and this budding checkpoint is MEC1 dependent (48). We synchronized log-phase wild-type and dap1Δ strains in G1 with α-factor and then released the cells from G1 arrest and followed progression of the cell cycle by analyzing budding morphology. Both wild-type and dap1Δ strains delayed budding when released into media containing MMS (Fig. 3A), suggesting that the cell cycle checkpoint is intact in dap1Δ cells. In contrast, mec1-21 cells did not delay budding under similar conditions (Fig. 3A).

We examined a molecular end point for the cell cycle checkpoint, monitoring the phosphorylation of the Rad53p checkpoint protein kinase. Following treatment with MMS, Rad53p becomes hyperphosphorylated (44, 51), and this phosphorylation is dependent on an intact Mec1p/Tel1p pathway (44). We used a strain with 13 copies of the Myc epitope tag integrated at the 3′ end of the genomic copy of RAD53; the epitope-tagged Rad53p was readily detectable in strains harboring the integrated tag (Fig. 3B, lanes 3 to 6) but was undetectable in cells harboring the native RAD53 allele (Fig. 3B, lanes 1 and 2). Treatment of wild-type cells with 0.05% MMS resulted in a marked increase in Rad53p phosphorylation, as expected (Fig. 3B, compare lanes 3 and 4), and the same shift in mobility was detected in dap1Δ cells (Fig. 3B, compare lanes 5 and 6). Thus, Dap1p is not required for Rad53p hyperphosphorylation following treatment with MMS. Using two separate criteria, we conclude that Dap1p is not required for the MMS-initiated cell cycle checkpoint.

Next, unsynchronized wild-type or dap1Δ cells were treated with MMS and cell cycle progression was determined by microscopic analysis of budding and by flow cytometry. We found that dap1Δ cells frequently develop an aberrant shmoo morphology when arrested with α-factor (R. J. Craven, unpublished observations). However, these elongated shmoo-shaped cells resembled budded cells, making the early stages of the cell cycle difficult to distinguish, so we resorted to analysis of unsynchronized cells.

In unsynchronized log-phase cultures, the cell cycle profiles of wild-type and dap1Δ cells were similar. Following 2 h of MMS treatment, the vast majority of wild-type cells had small to medium-sized buds (Fig. 4A), while dap1Δ cultures began to accumulate unbudded cells (Fig. 4B). The difference in unbudded cells between wild-type and dap1Δ cells following 3 h of MMS treatment was significant (P = 0.002) when analyzed by the Student t test. Failure to bud indicates an arrest in the G1 phase of the cell cycle. Although the G1-arrested cells failed to
One explanation for the accumulation of dap1Δ cells in G1 following MMS exposure is that Dap1p is required for adaptation to MMS exposure. Following prolonged damage, cells can override cell cycle arrest and continue to grow, a process called adaptation (54). If Dap1p is required for adaptation to a checkpoint, we expected that loss of Dap1p in combination with loss of checkpoint function would suppress the G1 arrest. To test this idea, we constructed a strain lacking both DAP1 and the RAD9 checkpoint gene, and we found that dap1Δ rad9Δ cells had an even stronger G1 arrest than either single mutant (Fig. 4C). We conclude that loss of Dap1p function inhibits cell cycle progression in G1 following MMS-induced damage and that this arrest is not due to an inability to adapt to the Rad9p-mediated checkpoint.

**Dap1p regulates telomere length.** A number of genes required for damage repair also regulate telomere length. We analyzed the telomeres of dap1Δ strains by Southern blotting. Telomeres in the dap1Δ mutant were elongated by approximately 100 bp compared to those in wild-type strains (Fig. 5, lane 3). While there was a small effect on telomere length in dap1Δ strains, telomeric silencing in dap1Δ strains was unaffected.

Dappe is a high-molecular-weight protein kinase related to the human Atm protein, and Tel1p is a central regulator of telomere length. To test whether DAPI and TEL1 are in the same genetic pathway, we compared telomere lengths of dap1Δ tel1Δ double mutants with those of the single dap1Δ and tel1Δ mutants. A combined dap1Δ tel1Δ mutant had telomeres that were slightly longer than those of the comparable tel1Δ strain (Fig. 5, compare lanes 2 and 4). In general, combined mutations in two genes in the same genetic pathway result in a phenotype of one or the other single mutant. Because the dap1Δ tel1Δ phenotype was not intermediate between those of the dap1Δ and tel1Δ single mutants, we conclude that TEL1 is epistatic to DAPI and that they function in the same genetic pathway.

Cells lacking DAPI are sensitive to drugs inhibiting sterol synthesis. Microarray analyses demonstrated altered expression of the DAPI transcript either in strains with repressed ERG11 expression or in strains treated with the ergosterol inhibitor itraconazole (26). ERG11 encodes lanosterol C-14 demethylase (29), a key enzyme in ergosterol synthesis. Erg11p is inhibited byazole compounds, which inhibit Erg11p by forming a complex with the heme iron component in the cytochrome group (Fig. 6) (55, 58). In addition, the homology

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**FIG. 2.** Dap1p regulates the response to MMS-induced damage. Log-phase cells were diluted 1:10 and spotted onto YPD plates (upper panel), YPD plates containing 0.01% MMS (middle panel), or YPD plates containing 20 mM hydroxyurea (HU) (lower panel). The strains tested were wild-type (RCY409-2a, top rows), dap1Δ (RCY409-4b, second rows), and dun1Δ (RCY409-5c, third rows). Strains harboring the dap1Δ mutation grew poorly on hydroxyurea and did not grow on plates containing MMS. The dun1Δ strain RCY409-5c is included as a damage-sensitive control strain.
between Dap1p and known steroid binding proteins (Fig. 1) suggested that Dap1p might regulate sterol synthesis in yeast.

We tested the ability of dap1/H9004 mutants to grow on itraconazole by directly applying the drug to the surfaces of YPD plates, allowing them to dry, then spotting series of 10-fold dilutions to the plates. Wild-type cells grew readily on plates coated with 0.1 mg of itraconazole per ml, while dap1/H9004 mutants did not (Fig. 6B, middle panel, top two rows). Itraconazole sensitivity is not a general property of damage checkpoint mutants, because rad9/H9004 mutants grow readily on itraconazole. Interestingly, a mec1-21 mutant strain was moderately sensitive to itraconazole, although the reason for this is unclear, as no sterol synthetic phenotypes have been attributed to mec1 mutants. A second Erg11p inhibitor, fluconazole, was also toxic to dap1Δ strains (Fig. 6B, lower panel, middle row).

Partial loss of sterol synthesis in dap1Δ mutants. Because dap1Δ cells were sensitive to itraconazole, we analyzed the profile of sterols in dap1Δ cells by gas chromatography. In dap1Δ cells, we detected a 9% decrease in overall sterol concentrations, with a 5% decrease in ergosterol and 43 and 34% decreases in the ergosterol intermediates zymosterol and fecosterol, respectively (Fig. 7). In addition, the ergosterol intermediate 4,4-dimethylzymosterol was undetectable in dap1/H9004 cells but was present at 3.3% in wild-type cells. In contrast, there was a 17.5% increase in squalene and a 4.7-fold elevation in the ergosterol intermediate lanosterol (eluting at 17.948 min in Fig. 7B). For these analyses, each sample was injected twice and the value reported for each sterol quantity was the average of two injections. Lanosterol metabolism is the target of theazole inhibitors itraconazole and fluconazole. We conclude that dap1/H9004 strains have a partial lesion in sterol synthesis that leads to decreases in products downstream of Erg11p (lanosterol-14-demethylase).

Membrane permeability in dap1Δ strains. Loss of ergosterol synthesis can lead to elevated membrane permeability. Mutants lacking the ERG6 gene have a 35-fold increase in uptake of crystal violet compared to wild-type strains at low temperatures, although the elevation is modest at 30°C (2). We examined whether the sensitivity of dap1Δ strains to MMS could be due to altered membrane permeability. In principal, ele-
FIG. 4. Dap1p regulates cell cycle progression following DNA damage. (A and B) Unsynchronized wild-type (A) or \textit{dap1}/H9004 (B) cells were treated with 0.05\% MMS and then fixed and counted at the time points indicated. Cells were scored microscopically as unbudded (left bars), small budded (center bars), or doublets (right bars). In \textit{dap1}/H9004 cells, there was a twofold increase in the number of unbudded cells and a sixfold increase in the percentage of large-budded cells within 2 h of MMS addition. The strains analyzed were RCY409-2a (wild type) and RCY409-4b (\textit{dap1}/H9004).

(C) The budding profiles of four different strains were compared at 2 h following the addition of 0.05\% MMS. The strains were RCY409-2a (wild type), RCY409-4b (\textit{dap1}), RCY406-1d (\textit{rad9}), and RCY406-5a (\textit{dap1 rad9}). Deletion of the \textit{RAD9} gene did not reverse the G\textsubscript{1} arrest observed in \textit{dap1} mutants following MMS-induced damage. Error bars indicate standard deviations.
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Dun1p may be involved in maintaining mitochondrial function.

Dun1p regulates mitochondrial maintenance. Mutant dap1Δ cells have elevated levels of petite colony formation (Table 4). The W303 strain background contains the ade2 mutation, which causes a red colony color due to the accumulation of adenine precursors. Cells that have lost the ability to respire grow as small white colonies and are inviable on plates containing a nonfermentable carbon source such as glycerol (12). Plate cultures of dap1Δ isolates contained increased numbers of small, white colonies. We measured the frequency with which these white colonies emerge in the population and found that dap1Δ strains have a 4.4-fold elevation in petite formation (19.6%, compared to 4.5% for the wild-type strain [Table 4]). Thus, Dap1p is required for wild-type levels of mitochondrial maintenance. The elevated level of petite formation may be due to the partial arrest of ergosterol synthesis in dap1Δ strains, because other ergosterol mutants, such as erg3 and erg6 mutants, have elevated petite formation (45; M. Bard, unpublished observations).

Petite colony formation is also characteristic of strains with mutations within RNR genes (13). Dun1p is required for transcriptional activation of RNR genes in response to damage (59), and dun1Δ mutants are damage sensitive, have elevated rates of petite formation (16) and mitotic recombination (15), and have diminished telomeric silencing (8). We used a dun1Δ strain as a positive control for elevated petite colony formation, and as expected, dun1Δ strains had an elevated level of petite colonies (Table 4).

These results suggest that Dap1p regulates mitochondrial stability. Petite colonies can arise through loss of mitochondrial biogenesis or through mutations in nuclear genes required for mitochondrial maintenance. Thus, it was possible that dap1Δ strains lose mitochondria because of an elevated mutation frequency in the nuclear genome. We measured the

Wild-type and dap1Δ strains were grown to log phase and incubated with 5 μg of crystal violet per ml at four different temperatures. The cells were then centrifuged, and the concentration of crystal violet in the supernatant was measured and compared with the beginning concentration to calculate uptake. Surprisingly, dap1Δ cells had a decreased crystal violet uptake relative to wild-type strains at each of the temperatures analyzed (Table 2), ranging from 1.3- to 13-fold lower than in the wild type. Our levels of uptake for the wild-type strain W303 were similar to those reported previously for the wild-type strain A184D at each of the temperatures analyzed (2). At all temperatures tested, the uptake of crystal violet in dap1Δ strains was significantly lower than that in wild-type cells, suggesting alterations in the endocytic pathway or transmembrane diffusion in dap1Δ mutants. To test whether dap1Δ strains are defective in uptake of compounds other than crystal violet from the media, we plated wild-type and dap1Δ strains on plates containing the selective drug Geneticin. Both wild-type and dap1Δ strains were sensitive to Geneticin, indicating that under physiological conditions, dap1Δ strains are capable of transporting compounds to the interior of the cell.

We then measured the sensitivity of dap1Δ strains to a cationic pulse. Cells were exposed to 2 M solutions of the chloride salts of calcium, chromium, potassium, magnesium, and nickel for 10 min and then plated for viability. Mutants with hyper-permeable membranes are sensitive to a pulse of cations at high concentrations (2). We found that the response of dap1Δ strains to these five cations was indistinguishable from that of wild-type strains (Table 3). We conclude that the altered sterol composition of dap1Δ strains does not disrupt the function of the cell membrane and does not allow a major influx of dyes or salts. Thus, it is unlikely that dap1Δ strains are sensitive to MMS purely because of overly permeable membranes, in contrast to phenotypes associated with the erg6 mutation.

FIG. 5. DAP1 regulates telomere length. Southern blot analysis of telomere lengths in the strains RCY93-7c (wild type) (lane 1), RCY23 (tell) (lane 2), RCY93-3c (dap1) (lane 3), and RCY93-2a (dap1 tell) (lane 4) is shown. In all cases, DNA was purified according to standard protocols, digested with PstI, blotted, and probed with a labeled fragment of the Y′ subtelomeric repeat. Migration of molecular size standards is indicated to the left.

vated membrane permeability could lead to increased local concentrations of MMS, causing a greater loss of viability than in wild-type strains.

Wild-type and dap1Δ strains were grown to log phase and incubated with 5 μg of crystal violet per ml at four different temperatures. The cells were then centrifuged, and the concentration of crystal violet in the supernatant was measured and compared with the beginning concentration to calculate uptake. Surprisingly, dap1Δ cells had a decreased crystal violet uptake relative to wild-type strains at each of the temperatures analyzed (Table 2), ranging from 1.3- to 13-fold lower than in the wild type. Our levels of uptake for the wild-type strain W303 were similar to those reported previously for the wild-type strain A184D at each of the temperatures analyzed (2). At all temperatures tested, the uptake of crystal violet in dap1Δ strains was significantly lower than that in wild-type cells, suggesting alterations in the endocytic pathway or transmembrane diffusion in dap1Δ mutants. To test whether dap1Δ strains are defective in uptake of compounds other than crystal violet from the media, we plated wild-type and dap1Δ strains on plates containing the selective drug Geneticin. Both wild-type and dap1Δ strains were sensitive to Geneticin, indicating that under physiological conditions, dap1Δ strains are capable of transporting compounds to the interior of the cell.

We then measured the sensitivity of dap1Δ strains to a cationic pulse. Cells were exposed to 2 M solutions of the chloride salts of calcium, chromium, potassium, magnesium, and nickel for 10 min and then plated for viability. Mutants with hyper-permeable membranes are sensitive to a pulse of cations at high concentrations (2). We found that the response of dap1Δ strains to these five cations was indistinguishable from that of wild-type strains (Table 3). We conclude that the altered sterol composition of dap1Δ strains does not disrupt the function of the cell membrane and does not allow a major influx of dyes or salts. Thus, it is unlikely that dap1Δ strains are sensitive to MMS purely because of overly permeable membranes, in contrast to phenotypes associated with the erg6 mutation.

Dap1p regulates mitochondrial maintenance. Mutant dap1Δ cells have elevated levels of petite colony formation (Table 4). The W303 strain background contains the ade2 mutation, which causes a red colony color due to the accumulation of adenine precursors. Cells that have lost the ability to respire grow as small white colonies and are inviable on plates containing a nonfermentable carbon source such as glycerol (12). Plate cultures of dap1Δ isolates contained increased numbers of small, white colonies. We measured the frequency with which these white colonies emerge in the population and found that dap1Δ strains have a 4.4-fold elevation in petite formation (19.6%, compared to 4.5% for the wild-type strain [Table 4]). Thus, Dap1p is required for wild-type levels of mitochondrial maintenance. The elevated level of petite formation may be due to the partial arrest of ergosterol synthesis in dap1Δ strains, because other ergosterol mutants, such as erg3 and erg6 mutants, have elevated petite formation (45; M. Bard, unpublished observations).

Petite colony formation is also characteristic of strains with mutations within RNR genes (13). Dun1p is required for transcriptional activation of RNR genes in response to damage (59), and dun1Δ mutants are damage sensitive, have elevated rates of petite formation (16) and mitotic recombination (15), and have diminished telomeric silencing (8). We used a dun1Δ strain as a positive control for elevated petite colony formation, and as expected, dun1Δ strains had an elevated level of petite colonies (Table 4).

These results suggest that Dap1p regulates mitochondrial stability. Petite colonies can arise through loss of mitochondrial biogenesis or through mutations in nuclear genes required for mitochondrial maintenance. Thus, it was possible that dap1Δ strains lose mitochondria because of an elevated mutation frequency in the nuclear genome. We measured the

FIG. 5. DAP1 regulates telomere length. Southern blot analysis of telomere lengths in the strains RCY93-7c (wild type) (lane 1), RCY23 (tell) (lane 2), RCY93-3c (dap1) (lane 3), and RCY93-2a (dap1 tell) (lane 4) is shown. In all cases, DNA was purified according to standard protocols, digested with PstI, blotted, and probed with a labeled fragment of the Y′ subtelomeric repeat. Migration of molecular size standards is indicated to the left.
rate of mutation at the CAN1 locus in dap1Δ strains in duplicate assays of 20 independent cultures. We did not detect a significant elevation in mutation frequency in dap1Δ cells compared to wild-type strains (2.8 × 10⁻⁷ in dap1Δ cells versus 2.4 × 10⁻⁷ in wild-type cells) (10).

DISCUSSION

We have found that the Dap1p protein is required for the damage response, telomere length maintenance, mitochondrial biogenesis, and sterol regulation. Mutants lacking Dap1p are particularly sensitive to the methylating agent MMS. MMS methylates DNA, causing the replication fork to stall during S phase, leading to double-stranded DNA breaks. Following cell cycle arrest and repair of the broken DNA, the cell cycle resumes. Our data indicate that yeast requires Dap1p in order to progress through the G1-S phase transition following MMS-induced damage.

Another interpretation of these data is that MMS induces a second type of damage aside from induction of double-stranded DNA breaks and that Dap1p is required for the G1-S transition following this type of damage. One potential secondary type of damage could arise from MMS-induced oxidative stress, which is associated with MMS (19). We pursued the possibility that MMS-induced oxidative stress might cause cell cycle arrest by damaging the cellular pool of sterols. Thus, the requirement for Dap1p to exit G1 might be related to the “sparking” requirement for ergosterol, in which diminished ergosterol levels prevent the G1-S transition (11). To test this idea, we treated wild-type cells for 3 h with various doses of itraconazole and then plated the cells on MMS. Surprisingly, itraconazole pretreatment had no effect on damage responsiveness in wild-type cells. This suggests that decreased sterol synthesis coupled with MMS-induced damage does not lead to an irreversible G1 arrest.

Cells exposed to chronic damage arrest and repair the damage. If the damage persists, cells attempt to override the arrest checkpoint in a process called adaptation (54). Our results indicate that it is unlikely that Dap1p functions by overriding a Rad9p-mediated G1 checkpoint. We prefer a model in which Dap1p performs an essential function in the G1-S transition following MMS-induced damage. The molecular function of Dap1p in exiting G1 after damage is not clear. However, Dap1p binds indirectly to a network of proteins that regulate cell cycle progression and the G1-S phase transition (25). We propose that protein interactions between Dap1p and its binding partners are required for the G1-S phase transition following MMS-induced damage.

We have shown that Dap1p is required for wild-type ergosterol levels. Ergosterol is a key component of the yeast cell membrane and resembles cholesterol structurally. Ergosterol levels regulate membrane fluidity and permeability, endocyt-
sis, secretion, vacuole fusion, mitochondrial respiration, oxygen sensing, gene expression (50), and a cell cycle sparking function (reviewed in reference 11). We have demonstrated that dap1 strains are sensitive to azole inhibitors of ergosterol synthesis and have increased levels of sterol intermediates. However, dap1 mutants are distinct from erg mutants in that dap1 is not a sterol auxotroph, and dap1 mutants are capable of synthesizing ergosterol, albeit at reduced levels compared to the wild type.

Proteins that direct the synthesis of ergosterol have been previously implicated in damage repair. Deletion of the ERG3 genes can lead to increased membrane permeability, as shown in Table 2 and Figure 7. Dap1p regulates sterol synthesis in yeast. Gas chromatography sterol accumulation profiles in the wild-type (RCY409-2a) (A) and dap1 (RCY409-4b) (B) strains from the same genetic cross are shown. The peaks for squalene (sq), zymosterol (zy), ergosterol (erg), lanosterol (la), and 4,4-dimethylzymosterol (dz) are indicated. Loss of Dap1p led to an increase in squalene and lanosterol and a decrease in zymosterol and ergosterol.

### Table 2. Membrane permeability in dap1Δ cells determined by crystal violet uptake

| Strain           | Uptakea at the following temp (°C): |
|------------------|-------------------------------------|
|                  | 0        | 22       | 30       | 37       |
| Wild type (RCY409-2a) | 6.5 ± 0.8 | 24.9 ± 2.4 | 23.6 ± 1.3 | 31.8 ± 0.8 |
| dap1Δ (RCY409-2b)                  | 0.5 ± 0.5b | 16.2 ± 2.1b | 18.1 ± 1.0b | 25.3 ± 1.4b |

a Crystal violet uptake was measured as described by Bard et al. (2) and in Materials and Methods. Cells were incubated with crystal violet for 10 min at the various temperatures and centrifuged. The $A_{opt}$ of the supernatant ($A_s$) was measured and compared with the absorbance of the starting solution ($A_i$); percent uptake equals ($A_i - A_s$)/$A_i$. The results from four separate reactions were averaged, and the standard deviation is shown.

b Significantly different from the result for the wild-type strain by the Student $t$ test ($P < 0.05$).
gene, encoding C-5 sterol desaturase, leads to MMS sensitivity, and erg28 and arv1 strains are MMS sensitive, but to a lesser extent (3). In addition, erg6 mutants are sensitive to the chemotherapeutic agents daunomycin and doxorubicin (24). It is likely that some of these strains are sensitive to MMS because of increased membrane permeability (2), increasing the effective concentration of damaging agent in the cell. However, we found that dap1 strains do not have elevated membrane permeability by two separate assays, indicating that the deficiency of dap1 strains in damage repair goes beyond simple changes in membrane structure. Interestingly, loss of ergosterol synthesis has been linked to decreased endocytosis in some mutants (23), consistent with the decreased uptake of crystal violet in dap1Δ cells.

Mutants lacking Dap1p are defective in mitochondrial mitochondrial biogenesis. We propose that this phenotype is due to abnormal ergosterol synthesis in dap1Δ cells. The relationship between ergosterol synthesis and mitochondrial regulation is poorly understood, because ergosterol is not a major constituent of mitochondrial membranes relative to other membranes. However, several ergosterol synthetic mutants have decreased mitochondrial function, including erg3 (45) and erg6 (unpublished observation) strains. In addition, overexpression of the mitochondrial COX3 gene confers resistance to azole compounds (31), further suggesting a link between altered respiration and ergosterol regulation.

Dap1p is homologous to the MAPR family. The term MAPR is based on the properties of individual family members, because only one member is a known membrane protein and only one has probable progesterone binding activity. The porcine MAPR was isolated biochemically from liver membrane extracts based on its binding to tritiated progesterone and only one has probable progesterone binding activity. The human MAPR homologue (also called 25-Dx) was subsequently cloned based on its reduced expression during lordosis, a female rodent mating behavior, and its negative regulation by progesterone (30). Green fluorescent protein-tagged murine 25-Dx localized to the cell periphery (30), and a number of MAPF family members have putative membrane-spanning sequences near their amino termini. The homology between Dap1p and membrane-associated progesterone binding proteins is consistent with a similar localization for Dap1p. Because MAPR proteins are putative progesterone binding proteins, we examined the toxicity of progesterone to strains lacking Dap1p but detected no difference between dap1Δ and wild-type cells with respect to progesterone-mediated toxicity.

Dap1p is the first member of the MAPR family to be directly linked to the response to damage and sterol synthesis. Some of these functions are probably conserved in other organisms. DAP1 homologues are present in many model organisms, including budding and fission yeasts, Drosophila, C. elegans, Arabidopsis, and mice. Our results also indicate that an improved understanding of this gene family may lead to novel approaches for antifungal compounds or strategies to increase the efficacy of existing antifungal agents.

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### TABLE 3. Membrane permeability in dap1Δ cells determined by viability after a cation pulse

| Strain       | % Viability after exposure to: | Ca2⁺ | Cr⁺ | K⁺ | Mg2⁺ | Ni⁺ |
|--------------|--------------------------------|------|-----|----|------|-----|
| Wild type (RCY409-2a) | 96 ± 8 18 ± 3 86 ± 6 91 ± 8 80 ± 17 |      |     |    |      |     |
| dap1Δ (RCY409-2b) | 80 ± 11 22 ± 5 94 ± 4 93 ± 3 88 ± 18 |      |     |    |      |     |

* Viability compared to that in a saline control after 10 min of exposure to 2 M solutions of the various chloride salts. Values reported are the means of three separate determinations ± standard deviations, and all determinations were repeated one to three times.

### TABLE 4. Mitochondrial stability in dap1Δ cells

| Strain (genotype) | % Petite colonies (mean ± SD) | Avg % petite colonies (fold vs wild type) |
|------------------|------------------------------|-----------------------------------------|
| R CY407-12d (wild type) | 6.1 ± 1.3, 2.8 ± 3.6 | 4.5 (1) |
| R CY407-1d (dap1Δ) | 16.9 ± 7.4, 22.3 ± 8.8 | 19.6 (4.4)* |
| R CY409-5c (dun1Δ) | 16.1 ± 5.6, 11.8 ± 4.6 | 14.0 (3.1)* |

* Twenty separate colonies were picked, diluted, and then plated on YPD plates. After 3 days of growth at 30°C and an additional 3 days of incubation at 4°C, the numbers of red and white colonies were counted, and the percentage of small, white colonies was calculated. One hundred white colonies from each genotype were then patched onto medium containing gleyeol as a carbon source to confirm that the colonies were petite.

* Results from two 20-plate assays are shown.

* Statistically significant (t test; P < 0.05) difference compared to wild-type cells.
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