Activation of budding yeast replication origins and suppression of lethal DNA damage effects on origin function by ectopic expression of the co-chaperone protein Mge1

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running title: lethal effects of DNA damage and ORC function
Summary

Initiation of DNA replication in eukaryotes requires the Origin Recognition Complex (ORC) and other proteins that interact with DNA at origins of replication. In budding yeast, the temperature-sensitive orc2-1 mutation alters these interactions in parallel with defects in initiation of DNA replication and in checkpoints that depend on DNA replication forks. Here we show that DNA-damaging drugs modify protein-DNA interactions at budding yeast replication origins in association with lethal effects that are enhanced by the orc2-1 mutation or suppressed by a different mutation in ORC. A dosage suppressor screen identified the budding yeast co-chaperone protein Mge1p as a high copy suppressor of the orc2-1-specific lethal effects of adozelesin, a DNA-alkylating drug. Ectopic expression of Mge1p also suppressed the temperature sensitivity and initiation defect conferred by the orc2-1 mutation. In wild type cells, ectopic expression of Mge1p also suppressed the lethal effects of adozelesin in parallel with the suppression of adozelesin-induced alterations in protein-DNA interactions at origins, stimulation of initiation of DNA replication, and binding of the precursor form of Mge1p to nuclear chromatin. Mge1p is the budding yeast homologue of the E. coli co-chaperone protein GrpE, which stimulates initiation at bacterial origins of replication by promoting interactions of initiator proteins with origin sequences. Our results reveal a novel, proliferation-dependent cytotoxic mechanism for DNA-damaging drugs that involves alterations in the function of initiation proteins and their interactions with DNA.

Keywords: Origin recognition complex/anticancer drugs/initiation of DNA replication/programmed cell death/chaperone proteins
Introduction

Initiation of DNA replication is regulated by protein complexes assembled at origins of replication, the sites on chromosomes where DNA synthesis begins. In *E. coli*, initiation complexes are nucleated by the initiator protein DnaA, which interacts with specific sequences at *oriC*, the *E. coli* chromosomal origin of replication. DnaA also interacts with and regulates origins of replication of several bacterial viruses and plasmids (1). In eukaryotic cells, the assembly of initiator protein complexes occurs in G1 and requires interactions between the highly conserved six-subunit Origin Recognition Complex (ORC) and/or other proteins and DNA (reviewed in (2)). Although the specificity of these protein-DNA interactions varies in different organisms or at different developmental stages, they are critically important to origin function.

In the budding yeast *S. cerevisiae*, the temperature-sensitive *orc2-1* mutation alters the structure of ORC and its interactions with DNA in parallel with defects in initiation of DNA replication ((3,4); P. Trabold and W. Burhans, unpublished observations). The *orc2-1* mutation also causes defects in checkpoints that depend on ORC to establish DNA replication forks (5-7). When shifted to non-permissive temperatures, the effects of the *orc2-1* mutation are lethal in cells synchronized in G1 before the temperature shift, but much less lethal in cells first synchronized in S phase, and mitotic cells or non-cycling cells in G0 are refractile to these effects (3,5). The lethality of the *orc2-1* mutation is caused by inhibition of origin licensing in G1 and subsequent entry into S phase and mitosis with defective checkpoints and insufficient replication forks to completely replicate the genome. This induces DNA damage and an apoptotic phenotype that
includes production of reactive oxygen species (ROS) and activation of a budding yeast metacaspase (Weinberger et al., submitted).

A similar cell cycle-specific and proliferation-dependent pattern of lethality occurs in wild type budding yeast and human cells treated with adozelesin (8), an experimental DNA-alkylating antitumor drug that inhibits initiation of DNA replication in both these organisms (5,8). In budding yeast, this pattern of lethality is exacerbated by the orc2-1 mutation at semi-permissive temperatures – that is, the orc2-1 mutation increases sensitivity to adozelesin when cells are synchronized in G1 before treatment, and orc2-1 cells in other phases of the cell cycle or in G0 are less sensitive to the lethal effects of this drug (5). This contrasts with the S phase-specific lethal effects of the DNA-alkylating agent methyl methanesulfonate (MMS) that have been reported in checkpoint-defective mecl and rad53 cells (9). Furthermore, the orc1-161 mutation, which also causes defects in initiation and in S phase checkpoints, suppresses, rather than enhances, the G1-specific lethal effects of adozelesin (5). These findings indicate that one component of adozelesin-induced lethality in budding yeast is related to effects on ORC function specifically in G1 cells that are only indirectly related to the requirement for ORC in S phase checkpoints.

Many other DNA-damaging drugs exert similar proliferation-dependent and cell cycle-specific lethal effects in mammalian cells (10). The nature of the relationship between the cell cycle and sensitivity to these cytotoxic agents remains unclear. A primary function of ORC in G1 cells of both mammals and yeast is to establish pre-replicative complexes (“pre-RCs”) that “license” origins of replication for subsequent
initiation of DNA replication in S phase (11). The absence of origin licensing in G0 or mitosis (12) and conservation of the licensing mechanism from yeast to mammals suggested the hypothesis that some of the G1-specific and proliferation-dependent cytotoxic effects of adozelesin might be related to inhibition of pre-RC-dependent origin licensing (5). Consistent with this hypothesis, in both budding yeast and mammals, adozelesin induces the proteasome-dependent degradation of Cdc6, which interacts with ORC and is required for origin licensing in all eukaryotes (8).

To better understand the cytotoxic effects of adozelesin and other DNA-damaging drugs, in this study we further characterized the ORC-related effects of adozelesin on viability in budding yeast. As part of this effort, we performed a dosage suppressor screen to identify \textit{S. cerevisiae} genes that, when expressed at high levels, eliminate the increased sensitivity to adozelesin conferred by the \textit{orc2-l} mutation. This screen identified wild type Orc2p and the mitochondrial co-chaperone Mge1p as high dosage suppressors of the \textit{orc2-l}-dependent lethal effects of adozelesin. Overexpression of Mge1p also stimulated initiation of DNA replication in both \textit{orc2-l} and wild type cells and partly suppressed the sensitivity of wild type cells to adozelesin. DNase I footprinting revealed that adozelesin and the DNA alkylating agent methyl methanesulfonate (MMS) altered interactions of proteins with DNA at origins of replication, and that the altered interactions induced by adozelesin were suppressed by ectopic expression of Mge1p in concert with binding of the precursor form of this protein to nuclear chromatin. These findings reveal a novel proliferation-dependent cytotoxic mechanism for DNA-damaging drugs that involves alterations in the function of proteins that operate at origins of replication in G1 cells.
Since ORC and other initiation proteins are highly conserved, a similar mechanism may contribute to the lethal, apoptosis-inducing effects of DNA damaging drugs in proliferating mammalian cells.

**Experimental Procedures**

**Yeast Strains, Cell Cycle Synchronization, and Adozelesin Treatment:** The strains employed in this study are W303 (MATa ade2-1 ura3-1 his3-11 trp1-1 leu2-3, 112 can1-100), YB0057 (W303 orc2-1), OAY660 (MATa ura3-1 trp1-1 leu2-3, 112 his3-11, 15 can1-100 ade2-1 bar1Δ:hisG lys2Δ:hisG leu2::ORC1 (LEU2)) and OAY661 (OAY660 leu2::orc1-161 (LEU2)). The W303 and orc2-1 strains were a gift of Bruce Stillman, Cold Spring Harbor Laboratories, and the orc1-161 strain was a gift of Stephen Bell, M.I.T. Cells were grown in YPD medium or in YP galactose or YP raffinose in the case of strains engineered to express Mge1p from a galactose-inducible promoter after integration, or minimal medium + galactose or raffinose for strains expressing Mge1p from a plasmid. G1 arrest was obtained by culturing wild type cells for 3 hours with 3 μM α-factor (Invitrogen), or 12 μM α-factor in the case of orc2-1 cells. Cells were arrested in G2/M with 2.5-3 hour treatment with nocodazole (20 μg/ml; Sigma). Adozelesin (gift of Upjohn Pharmacia, Kalamazoo, MI) was added to medium of cells cultured at 23°C at indicated concentrations for 4 hours or at 4 μM for indicated times. All cell cycle arrests were monitored and confirmed by flow cytometry.

**In Vivo DNase I Genomic Footprinting:** The genomic footprinting technique was employed as described previously (13), with some modifications. Cells were grown at 25°C in YPD medium to a concentration of 1-1.5 x 10⁷ cells/ml and arrested in the appropriate cell cycle phase, and then were treated with either methyl methanesulfonate
(MMS; Sigma) or adozelesin. DNase I digestion with increasing amounts of enzyme occurred on ice for 6 minutes. Immediately after lysis of cells, NP-40 was added to a final concentration of 0.1%. DNA concentration was estimated by gel electrophoresis and comparison of ethidium bromide signals to 500 ng of BstEII digested λ DNA using AlphaImager 2200 v5.1 (Molecular Dynamics). Primer JD60 was used to detect the 2 µm origin (12). Primers were 5’ end-labeled with 32P. DNA sequencing samples were obtained using the Circumvent DNA sequencing kit (NEB). The samples were subjected to electrophoresis on a 6% sequencing gel, and the dried gel was exposed to a PhosphoImager screen for analysis (Molecular Dynamics).

Viability Assays: Cells were grown in YPD or minimal medium at the indicated temperature to a concentration of 1-2 x 10^7 cells/ml. Cell cycle arrests were performed as described above and monitored by flow cytometry. For each data point, cells were plated in triplicate on YPD at room temperature. Viability is normalized to the viability of the cell cycle arrest in the absence of drug treatment. The values presented are averages of at least 3 independent experiments.

Dosage Suppressor assay, cloning and strain construction: A yeast genomic library (ATCC 37323) was transformed into YB0057 (W303 orc2-1) and transformants were exposed to 4 µM adozelesin in minimal medium at 35°C for 4 hours. These conditions were chosen based on the fact that minimal medium and the elevated temperature maximized the lethal effects of adozelesin (first cycle survival was 0.2%). Survivors were cultured and similarly exposed to drug to produce 98.3% cell death. Preliminary characterization of the library sequences in the survivors of the second round of drug exposure was performed by probing Southern blots with a 622bp Sal1-HindIII YEp24
fragment, which flanks the site of insertion of the library sequences. One hundred and fifteen colonies were screened, but only three distinct patterns were seen in the Southern blots. Representatives of these groups were re-transformed into orc2-1 cells and treated with adozelesin to ensure that resistance required plasmid sequences. To identify the library inserts, the plasmids were partially sequenced using YEp24 sequences flanking the site of insertion of the library clones as primers.

The MGE1 gene and its native upstream control elements were amplified from one of these isolates by PCR and inserted into the 2µm-based pRS426 (14) to produce pMW492. A galactose-inducible MGE1-integrating plasmid (pMW509) was constructed by ligating a PCR-amplified MGE1 structural gene into the pLD3 plasmid backbone (15) at a BamH1 restriction site. PMW509 was linearized with NheI, transformed into W303 and integrants were selected for histidine prototrophy. Selected MGE1 integrants were verified by Southern blotting. pMW497 is an Orc2p-expressing plasmid that was constructed by excising ORC2 sequences from pJR1263 (gift of Jasper Rine, U.C. Berkeley) as a 2.8kb SacI fragment and ligating this fragment into pRS426.

**Chromatin experiments and neutral-neutral 2-D gels.** Crude nuclei were prepared from whole cells by the procedure described in (16). Briefly, spheroplasts were generated with zymolyase (Seikagaku, Inc. Japan) and were then disrupted with a dounce homogenizer and nuclei recovered by differential centrifugation. Crude nuclei were lysed by the addition of 1% triton X-100. Chromatin-associated and nuclear soluble fractions were separated by centrifugation as described by (17). Crude lysates were prepared by bead beating in 100mM NaCl, 25mM Tris-HCl, 10% Glycerol 0.1% Triton X-100 with one protease inhibitor tablet (Roche) per 5ml lysis buffer. 30 µg of protein from different
samples were size-fractionated on 12% PAGE gels, and blotted proteins were visualized by immunodetection using an ECL kit from Amersham. Anti-Mge1p (Laloraya et al., 1994) was a gift of Elizabeth Craig (University of Wisconsin) and used at a dilution of 1:2000; the secondary antibody was antirabbit at 1:7000 dilution. Anti-actin was used at 1:1000 and detected with antigoat diluted 1:2000. All other antibodies were obtained from Santa Cruz. Neutral-neutral 2-D gel analysis was performed as described previously (5) using cells transformed with the empty vector pRS426 or pMW492 expressing Mge1p and grown in minimal selective medium to maintain these plasmids.

Results

**ORC-related cytotoxic effects of adozelesin and MMS.** We first sought to further characterize the *orc2-1*-dependent sensitivity of budding yeast cells to adozelesin. The *orc2-1* mutation also causes greater sensitivity to MMS (9), and so we examined the effects of this compound as well. Treatment of budding yeast cells with adozelesin or MMS caused time- and dose-dependent cytotoxic effects that were exacerbated by the *orc2-1* mutation (Fig. 1A-D). In contrast, as reported earlier for adozelesin (5), both adozelesin and MMS effects on viability were reduced, rather than enhanced, by the *orc1-161* mutation (Fig. 1E and F). These findings establish the existence of DNA damage effects on viability that are directly related to ORC function.

To better understand the increased sensitivity of *orc2-1* cells to DNA damaging agents, we performed a dosage suppressor assay to search for genes that, when expressed at high levels, would suppress the increased sensitivity to adozelesin conferred by the
orc2-1 mutation. orc2-1 cells were transformed with a genomic library of budding yeast sequences cloned into a high copy-number vector and subjected to multiple rounds of selection for resistance to adozelesin. Of a large number of drug-resistant colonies selected for further characterization, most harbored a single library isolate expressing the wild type ORC2 gene. Analysis of adozelesin-induced lethality in cells re-transformed with this plasmid indicated that expression of Orc2p at high levels in orc2-1 cells suppressed the orc2-1-dependent sensitivity to adozelesin (Fig. 2A; “ORC2”). One resistant colony harbored a different plasmid expressing two genes. Transformation of orc2-1 cells with each of these genes separately cloned into plasmids identified one of them, MGE1, as an additional dosage suppressor of the sensitivity specifically conferred by the orc2-1 mutation (Fig. 2A; “MGE1”). High level expression of Mge1p also suppressed the sensitivity of wild type cells to adozelesin (Fig. 2B).

Most of the increased sensitivity to adozelesin of orc2-1 cells occurs in the G1 phase of the cell cycle, and suppression of adozelesin sensitivity by the orc1-161 mutation also occurs specifically in G1 (5). Mge1p suppression of orc2-1-dependent sensitivity was also greatest in G1 phase compared to mitosis or G0 (Fig. 2C). Furthermore, FACS analysis of DNA content in cells transformed with the empty vector control plasmid or a plasmid expressing Mge1p indicated that expression of high levels of Mge1p caused a slight increase in the number of cells in G1 phase (Fig. 2D), similar to an earlier report (18). Therefore, suppression of adozelesin cytotoxicity by Mge1p is not caused by redistribution of cells to phases of the cell cycle other than G1 where they are more refractile to adozelesin’s cytotoxic effects. orc2-1p is less stable than its wild type
counterpart (7). Suppression of *orc2-1* sensitivity to adozelesin was also not related to increased stability of orc2-1p associated with ectopic expression of Mge1p (data not shown).

*Binding of unprocessed Mge1 to chromatin in the nucleus and stimulation of initiation of DNA replication.* Mge1p is a budding yeast homologue of the *E. coli* co-chaperone GrpE. Mge1p cooperates with Ssc1p and Mdj1p, which are homologues of *E. coli* DnaK and DnaJ proteins, to unfold other proteins in order to facilitate their import into mitochondria (19,20). In bacteria, the GrpE/DnaK/DnaJ chaperone machinery modulates initiation of DNA replication in chromosomal, bacteriophage and plasmid replicons by modulating interactions of initiator proteins with origin sequences (reviewed in (21). Although Mge1p has not been shown to reside in the nucleus of budding yeast cells, this possibility has not been excluded. The GrpE/DnaK/DnaJ paradigm in bacteria and previously described alterations in protein-DNA interactions caused by the *orc2-1* mutation (4) suggested that suppression of *orc2-1*-dependent sensitivity to adozelesin by Mge1p might be related to its stabilization of initiator protein complex interactions with DNA.

To determine whether native or ectopically expressed Mge1p can be detected in the nucleus, immunoblots of total cell, nuclear soluble, and nuclear chromatin proteins isolated from wild type cells and cells that can express a large amount of Mge1p from a galactose-inducible promoter were probed with an antibody against Mge1p. Mge1p exists in two forms – a precursor protein with an apparent molecular weight of 26 kD
containing a leader sequence that targets this protein to mitochondria, and a processed form of apparent 21 kD produced by removal of the leader sequence in mitochondria. Although very little of either protein was detected by immunoblotting of whole cell lysates of wild type cells grown in either raffinose or galactose in short exposures of these blots to film (Fig. 3A; “wt-raf, wt-gal”), these same short exposures detected large amounts of Mge1p in lysates from two independently isolated colonies of cells transformed with an integrating plasmid harboring gal-inducible MGE1 when these cells were grown in galactose (Fig. 3A; “#9-gal, #12-gal”), but not raffinose (Fig. 3A; “#9-raf, #12-raf”). However, the amount of the larger, unprocessed Mge1p was significantly greater than the processed form of Mge1p in extracts of cells from the #9 compared to the #12 isolate. When grown in galactose, cells from the #9 isolate were also less sensitive to adozelesin compared to cells from the #12 isolate (Fig. 3B).

Approximately equal amounts of galactose-induced processed and unprocessed Mge1p were also detected in nuclear extracts isolated from the #9 isolate cells (Fig. 3C; “#9 NE”). Since the precursor form of Mge1p is processed in mitochondria, its presence in nuclear extracts may reflect significant contamination of nuclear preparations with mitochondria, as has been observed previously (16). In fact, when nuclei from #9 isolate cells ectopically expressing large amounts of both forms of Mge1p were fractionated into soluble proteins and proteins bound to chromatin (Fig. 3C; “chromatin”), most of the 21 kd processed Mge1 induced by galactose was found in the soluble (“s”) fraction rather than the chromatin pellet (“p”), and very little 26 kD unprocessed Mge1 was observed in the soluble fraction (Fig. 3B; “chromatin; gal”). In contrast, the chromatin pellet was
enriched for the unprocessed, but not the processed, form of Mge1p (Fig. 3C; “chromatin; gal”). As expected, chromatin pellets, but not supernates, from cells grown in either raffinose or galactose were also enriched for Orc2p (Fig. 3C; “orc2p”). The association of the unprocessed, and not the processed, mitochondrial-specific form of Mge1p with chromatin indicates that this association is specific, and is not related to contamination of chromatin preps with mitochondria or mitochondrial proteins. These results are consistent with a nuclear role for ectopically expressed unprocessed Mge1p in the suppression of the cytotoxic effects of adozelesin.

In the absence of DNA-damaging drugs, the initiation defect and reduced viability conferred by the orc2-1 mutation are associated with destabilized protein-DNA interactions at origins, even at semi-permissive temperatures (4). The bacterial GrpE/DnaK/DnaJ paradigm suggests that ectopically expressed Mge1p bound to chromatin in the nucleus might stabilize ORC interactions with DNA, which is expected to suppress the temperature sensitivity and initiation defect associated with the orc2-1 mutation. In fact, overexpression of Mge1p increased the viability of orc2-1 cells at the semi-permissive temperature of 30° C (Fig. 3D). To determine whether this was accompanied by suppression of the orc2-1 initiation defect, we examined the effect of Mge1p overexpression on DNA replication intermediates at ORI501 in orc2-1 cells at the semi-permissive temperature of 23°C using neutral-neutral 2-dimensional agarose gel electrophoresis (22). Passive replication of a region of DNA by a single replication fork emanating from an origin of replication outside this region is detected in these gels by a “fork arc” in restriction fragments from this region, and fragments that initiate DNA
replication (and thus have two replication forks) are detected in a “bubble arc” (Fig. 3E). Very few replication intermediates were detected at ORI501 in *orc2-1* cells transformed with an empty vector plasmid, and those that were detected were single replication forks passively replicating this locus (Fig. 3F; “*orc2-1* vector”). This is consistent with the relatively low initiation activity of ORI501 (23) and defective initiation caused by the *orc2-1* mutation. Significantly more replication intermediates were detected at ORI501 in *orc2-1* cells transformed with the high copy number plasmid expressing Mge1p, including initiation intermediates in a bubble arc (Fig. 3F; “*orc2-1 MGE1*”). Also detected at this locus under these conditions were single replication fork intermediates in the fork arc. The stronger signals detected from larger, later replicating forks compared to smaller, early replicating forks in this fork arc indicates that they correspond to initiation intermediates emanating from the off-center initiation site within the restriction fragment containing ORI501 that was analyzed in this experiment (Fig. 3E; “late forks” and Fig. 3F). Therefore, overexpression of Mge1p suppresses the defect in initiation of DNA replication in *orc2-1* cells.

Suppression of the sensitivity of wild type cells to adozelesin by Mge1p (Fig. 2B) suggested that Mge1p overexpression might also stimulate initiation in wild type cells. In the absence of ectopically expressed Mge1p, larger numbers of replication forks were detected passively replicating the ORI501 region in wild type cells compared to *orc2-1* cells (Fig. 3F; “wild type vector”). This reflects the relatively low activity of ORI501 and increased activation of adjacent replication origins in wild type cells compared to *orc2-1* cells due to the absence of an initiation defect. The absence of a detectable bubble arc at
ORI501 in wild type cells in this experiment is also related to less frequent initiation at this origin in cells grown in the defined medium required to maintain the Mge1p-expressing plasmid, since a bubble arc is clearly detected when these cells are grown in rich medium (data not shown). However, despite their growth in defined medium, significant numbers of initiation intermediates were detected in these cells when they were also expressing Mge1p (Fig. 3F; “wild type MGE1”). Therefore, in addition to suppressing the initiation defect in orc2-1 cells, overexpression of Mge1p stimulates initiation of DNA replication in wild type cells.

Adozelesin and MMS alter protein-DNA interactions at origins of replication.

These results are consistent with the possibility that overexpression of Mge1p stabilizes protein-DNA interactions at origins of replication, and that these interactions are destabilized by DNA-damaging drugs. To address this latter possibility, we employed DNaseI footprinting to ask whether MMS or adozelesin alter protein interactions with DNA at the 2 μm plasmid origin of replication. In the absence of drugs, ORC and/or other protein interactions with DNA are indicated by the detection of a prominent ORC-dependent DNase I hypersensitive site (“ORC HS”) adjacent to the 2 μm origin ARS consensus sequence (ACS) in cells arrested in mitosis with nocodazole (12). Since the orc2-1 mutation substantially reduces the signal from this hypersensitive site at semi-permissive temperatures in the absence of drugs (4), this experiment was performed only in wild type cells.
In the absence of drug treatment, size-fractionation of primer extension products obtained from DNase I-digested chromatin isolated from nocodazole-arrested cells revealed the ORC HS (Fig. 4A; “ORC HS” indicated by arrows) at a position in the 2 µm origin (determined by comparison with an adjacent sequence ladder) that was identical to the site reported previously (12). Treatment of cells with MMS caused a dose-dependent reduction in signals from the ORC HS (Fig. 4B). The changes in chromatin that caused the loss of this signal were not related to cell death, because the effect of MMS treatment on viability was minimal in mitotic-arrested cells employed in this experiment (greater than 95% survival). No other changes in chromatin structure induced by MMS treatment were detected in the region surrounding the ORC HS. The loss of the ORC HS in the absence of other detectable changes in chromatin was identical to the effects described previously for the orc2-1 mutation at a semi-permissive temperature in the absence of drugs (3,4). Therefore, like the orc2-1 mutation, MMS alters protein-DNA interactions at an origin of replication in budding yeast cells.

To determine whether adozelesin induces a similar change in chromatin structure at the 2 µm origin of replication that might be suppressed by ectopically expressed Mge1p, we performed DNase I footprinting analysis of adozelesin-treated and untreated cells from the #9 isolate described in Figure 3, which contain high levels of Mge1p when grown in galactose, but not raffinose. In the absence of adozelesin treatment, the ORC HS was clearly detected in nocadazole-arrested cells grown in either raffinose or galactose (Fig. 4C; “no drug”). Therefore, protein-DNA interactions at the 2 µm origin are not detectably altered by either of these growth conditions. Adozelesin treatment of
cells cultured in either raffinose or galactose produced signals from primer extension products in DNase I-digested chromatin that were not detected in chromatin isolated from untreated controls (Fig. 4C; open arrows, “adozelesin + raf”; adozelesin + gal”). However, these signals were not caused by DNase I cleavage at new chromatin hypersensitive sites associated with altered protein-DNA interactions, because they occurred in the absence of DNase I (Fig. 4C; “adozelesin”, lanes marked “0”) and were also observed in DNase I-digested naked DNA isolated from the same cells (Fig. 4C; “ND”). Adozelesin:DNA adducts block polymerase extension on defined DNA templates (24). Since these primer extension products were only detected in adozelesin-treated cells, they are likely caused by Vent polymerase pausing during the primer extension reaction at sites where adozelesin:DNA adducts have formed. In fact, direct measurements of adducts in 2 µm circle plasmid DNA indicated that 30-40% of these plasmids contained at least one adduct in the origin region (data not shown).

In addition to signals from primer extension products related to adozelesin adducts, the ORC HS was detected in chromatin isolated from adozelesin-treated cells that were not ectopically expressing Mge1p due to their growth in raffinose (Fig. 4C; “adozelesin + raf”, solid arrow). However, the intensity of the signal from the ORC HS was diminished in these cells compared to signals from the ORC HS and other signals in untreated controls. As was the case with MMS, the diminished signal from the ORC HS in adozelesin-treated cells was not related to cell death, which was minimal (less than 5%) under the conditions of this experiment. Therefore, similar to the effect of MMS treatment, adozelesin treatment alters protein-DNA interactions at the 2 µm origin.
However, the intensity of signals from the ORC-HS compared to other signals was not altered by adozelesin treatment when cells were grown in galactose to induce expression of Mge1p (Fig. 4C; “adozelesin + gal”). Quantitation of signals from the hypersensitive site and adozelesin:DNA adducts at three levels of DNase I digestion indicated that a significantly stronger signal from the hypersensitive site relative to these adducts was detectable in chromatin from adozelesin-treated cells grown in galactose compared to raffinose (Fig. 4D). Therefore, ectopic expression of Mge1p suppresses the changes in protein-DNA interactions at the 2 \( \mu \text{m} \) origin induced by adozelesin.

**Discussion**

Fundamentally important connections exist between cell cycle regulation and cell death that remain poorly understood. In mammals, these connections are suggested by frequent observations that proliferating cells are more sensitive to DNA-damaging and other cytotoxic agents compared to quiescent cells in G0 (10). Yeast cells that enter stationary phase or are starved of nitrogen enter a similar G0-like state, where they are also significantly less sensitive to a variety of stresses (reviewed in (25)), for reasons that remain unclear.

Previous application of a chemical genetics approach to understanding the cytotoxic effects of adozelesin suggested that the increased sensitivity of proliferating budding yeast cells to this drug depended in part on the ORC-dependent “origin licensing” mechanism that regulates initiation of DNA replication in proliferating cells, which is absent from cells in G0 (5). The reduced viability of \( \text{orc2-1} \) compared to wild type cells
treated with adozelesin or MMS reported here (Fig. 1) and previously (5,6) and the suppression of orc2-1-dependent cytotoxic effects by high levels of wild type Orc2p (Fig. 2A) clearly establish that the lethality of DNA damaging agents is exacerbated by reduced ORC function. Although the orc2-1 mutation causes defects in initiation and in S phase checkpoints (5-7), most of the increased drug-induced lethality associated with this mutation is unlikely a consequence of these defects, for two reasons. First, the increased sensitivity to adozelesin conferred by the orc2-1 mutation – and its suppression by the orc1-161 mutation - occur specifically in cells exposed to this agent while in G1, where origin licensing occurs, and not in S phase cells (5). This contrasts with the increased sensitivity in S phase, rather than G1, of checkpoint-defective mec1 and rad53 mutant cells exposed to low concentrations of MMS (9). Second, cells harboring the orc1-161 mutation also harbor defects in initiation of DNA replication and in S phase checkpoints (although we detected a defective S phase checkpoint response to adozelesin, but not hydroxyurea, in an earlier FACS assessment of checkpoint proficiency in orc1-161 cells (5), subsequent 2-D gel analysis indicated that, similar to orc2-1, mec1 and rad53 strains (6,26) late S origins are activated in orc1-161 cells blocked in early S phase with hydroxyurea, which indicates they also harbor a defective replication checkpoint (P. Trabold and W. Burhans, unpublished)). Despite their defects in initiation and checkpoints, orc1-161 cells are resistant, instead of sensitive, to adozelesin and MMS (Figs. 1E and F and (5)). This resistance clearly points to an effect of DNA damage on ORC function that is separable from the initiation and checkpoint defects associated with mutations in ORC in these two strains.
In the absence of DNA-damaging drugs, the \textit{orc2-1} mutation alters protein-DNA interactions at origins of replication in parallel with the defect in initiation caused by this mutation (4). Our footprinting experiments establish that protein-DNA interactions at origins are similarly altered in wild type budding yeast cells treated with adozelesin or MMS (Figs. 4). These alterations are not a consequence of cell death, because the experiments that detected them were performed in mitotic-arrested cells, which are refractile to lethal effects associated with DNA damage or loss of ORC function due to the \textit{orc2-1} mutation (5). Altered protein-DNA interactions could occur in \textit{cis} associated with DNA damage lesions in the vicinity of origins, such as the adozelesin adducts that are responsible for some of the chromatin-independent primer extension products detected by footprinting in the 2 µm origin (Fig. 4C). They may also occur in \textit{trans}, perhaps in association with the loss of the ORC-interacting protein Cdc6. Cdc6 is destroyed by the proteasome in budding yeast cells exposed to adozelesin (8) or MMS (W. Weinberger and W. Burhans, unpublished) at the doses employed in our footprinting experiments. The fact that Cdc6 can modulate ORC-DNA interactions in extracts from budding yeast (27) suggests that altered protein-DNA interactions at origins of replication induced by DNA-damaging drugs could be related to Cdc6 destruction.

The altered protein-DNA interactions induced by adozelesin in wild type cells were suppressed by ectopic expression of Mge1p (Fig. 4C) in concert with decreased sensitivity to this drug (Fig. 2B) and stimulation of initiation of DNA replication. It is important to emphasize that the increased survival conferred by ectopic expression of Mge1p in these cells was substantial. For example, at lower concentrations of adozelesin,
viability was increased from less than ~10-20% in the absence of ectopic Mge1p expression to ~40-80% in cells ectopically expressing this protein (Fig. 2B). In the absence of drugs, ectopic expression of Mge1p suppressed the temperature sensitivity (Fig. 3D) and initiation defect (Fig. 3E & F) conferred by the orc2-1 mutation as well.

How ectopic expression of Mge1p exerts these effects remains unclear. Like its bacterial homologue GrpE, Mge1p is a nucleotide exchange factor. The nucleotide exchange activity of Mge1p stimulates ATP hydrolysis by the mitochondrial protein Ssc1p, which is a budding yeast homologue of DnaK (28,29). Together with the mitochondrial DnaJ homologue Mdj1p, the Mge1p/Ssc1p/Mdj1p chaperone complex facilitates the import of proteins into mitochondria. Similar to mammals, mitochondria play important roles in cell death pathways in yeast (reviewed in (30,31)). In this context, it seems reasonable to expect that suppression of adozelen lethal effects on adzaelesin lethality by ectopic expression of Mge1p could be related to effects on mitochondrial function or biogenesis.

Less apparent is a mechanism by which altered mitochondrial function could explain how ectopically expressed Mge1p suppresses DNA damage effects on protein-DNA interactions at replication origins and stimulates DNA replication in the nucleus. In fact, binding of the Mge1p precursor to nuclear chromatin (Fig. 3) suggests a more direct effect that occurs independently of mitochondrial events. Interestingly, the bacterial homologues of Mge1 and its interacting partners (GrpE and its interacting partners DnaK and DnaJ) were first identified by mutations that prevented lambda phage DNA replication and significantly inhibited E. coli chromosomal DNA replication (reviewed in
It is likely that the GrpE/DnaJ/DnaK chaperone complex stimulates initiation of DNA replication in *E. coli* by modifying the three-dimensional structure of DnaA, RepA and other chromosomal and plasmid initiator proteins – all of which are functional homologues of ORC - in a manner that increases their specific binding activity to origin sequences (reviewed in (21)).

The GrpE/DnaJ/DnaK paradigm suggests that Mge1p bound to chromatin in our experiments cooperates with nuclear DnaK and DnaJ homologues to modify the structure of ORC and/or other origin proteins in a manner that promotes more stable interactions with DNA. Consistent with this possibility is the recent discovery that a budding yeast homologue of DnaK physically associates with Orc4p, a subunit of ORC that appears to be related evolutionarily to RepA in *E. coli* and Cdc6 in Archaea (33). Also consistent with this possibility is the fact that Mge1p can stimulate heterologous DnaK proteins (34), as well as numerous reports that implicate chaperone proteins in the regulation of initiation of DNA replication in eukaryotic viral genomes (35-38) (39). Although there are no prior reports of Mge1p within the nucleus of budding yeast cells, a recent global analysis of protein-protein interactions in budding yeast cells detected Mge1p in several protein complexes that also contained nuclear proteins, including the DNA replication protein Sld2p and a number of DNA repair proteins, such as Rad54p, Cka1p, Mgt1p and Hrr25p (40). This raises the possibility that Mge1p has a previously undetected nuclear function. Alternatively, promiscuous entry of ectopically expressed Mge1p into the nucleus when expressed at high levels may allow it to assume the function of an unidentified nucleotide exchange factor that stimulates nuclear DnaK bound to Orc4p.
Regardless of how Mge1p exerted its effects in our experiments, our results reveal a novel cytotoxic mechanism involving DNA damage-induced inhibition of origin function that phenocopies the effects of the *orc2-1* mutation at high temperatures. At these temperatures, the *orc2-1* mutation causes an acute failure to establish or maintain licensing complexes in G1. This leads to lethal effects in the subsequent S phase associated with a reduced number of replication forks and the simultaneous inability to completely replicate the genome or mount checkpoints that respond to incomplete replication (Weinberger et al., submitted). The similar changes in the footprint of protein-DNA interactions at the 2 µm origin induced at semi-permissive temperatures in *orc2-1* cells (12) and by DNA damage in wild type cells (Fig. 4) and the ability of Mge1p to suppress these changes in parallel with suppression of lethality and stimulation of initiation of DNA replication strongly suggests that, similar to the effects of the *orc2-1* mutation at high temperatures, the lethal effects of DNA damage in wild type cells are caused in part by inhibition of origin licensing in G1 cells. Since ORC is required for origin licensing and the *orc2-1* mutation destabilizes the subunit structure of ORC (41), exacerbation of lethal effects of DNA damage by the *orc2-1* mutation is likely related to these destabilizing effects. In contrast, the *orc1-161* mutation may suppress the lethal effects of DNA damage by a mechanism that increases the stability of ORC at the same time that it causes initiation and checkpoint defects via a mechanism different from that associated with the *orc2-1* mutation. Since origin licensing is no longer required in cycling cells once replication forks are established, cells in S phase or later stages of the cell cycle should be refractile to disruption of protein-DNA interactions at origins, as was
the case in our experiments. A similar lack of requirement for origin licensing in G0 cells should render these cells less sensitive to DNA damage through this hypothetical mechanism as well.

A related mechanism for proliferation-dependent lethal effects of DNA damage may operate in mammalian cells. As in budding yeast, the ORC-interacting protein Cdc6 is destroyed in mammalian cells exposed to lethal amounts of DNA-damaging and other cytotoxic drugs that induce apoptosis (8,42-47). Furthermore, apoptosis in mammalian cells can be inhibited by mutations that block Cdc6 destruction (43), which indicates the destruction of this protein plays a causal role in cell death pathways. The results reported here are consistent with our model that this role is related to inhibition of origin licensing.

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Figure Legends

Fig. 1. ORC-dependent effects of adozelesin and MMS on viability. (A and B) Wild type and orc2-1 cells grown and YPD and treated with either 4 µM adozelesin or 0.03% MMS at 27° C. for the indicated time. Viability was normalized to untreated controls. (C-F) Wild type, orc2-1, and orc1-161 cells were treated with adozelesin or MMS at the indicated doses for 4 hours at 27° C. Viability was normalized to untreated controls.

Fig. 2. Suppression of adozelesin effects on viability by overexpression of Mge1p. A. Effect of 4 hours adozelesin treatment on viability of orc2-1 cells growing in minimal medium at 30° C and transformed with a high copy number empty vector or a vector expressing the MGE1 or ORC2 genes. B. Effect of 4 hours adozelesin treatment on similarly transformed wild type cells under identical conditions. C. Cell cycle-specific effects of Mge1p overexpression on viability of orc2-1 cells growing in minimal medium and treated with 4 µM adozelesin for four hours at 30° C. Cells were blocked in G1 with alpha factor, G2 with nocodazole, or driven into G0 by nitrogen starvation before addition of adozelesin (see Materials and Methods). D. Effect of overexpressing Mge1p on the cell cycle as determined by FACS measurement of DNA content.
**Fig. 3** Nuclear localization of overexpressed Mge1p and its stimulation of DNA replication. A. Western blots of Mge1p in lysates of wild type cells or cells expressing or not expressing Mge1p from a galactose-inducible promoter. #9 and #12 are two independent isolates of cells transformed with a galactose-inducible MGE1 gene. “u” is unprocessed Mge1p and “p” is Mge1p processed in mitochondria. raf = raffinose; gal = galactose. B. Suppression of adozelesin lethality in cells from the #9 and #12 isolates. Cells were treated with 4 mm adozelesin for indicated times and viability was measured by a colony-forming assay. Viability is normalized to that of untreated controls. C. Binding of the unprocessed form of Mge1p to chromatin in isolate #9 nuclei. “NE” is nuclear extract. “r” and “raf” = raffinose; “g” and “gal” = galactose; “p” = chromatin pellet; “s” = supernatant; NE = nuclear extract. D. Partial suppression of sensitivity to adozelesin by ectopically expressed Mge1p in the #9 and #12 isolates. Cells were exposed to 4 µM adozelesin for the indicated times and then assessed for survival by colony-forming assays. Values are normalized to untreated controls. E. Schematic of replication intermediates separated on neutral-neutral 2-D gels. F. Neutral-neutral 2-D gel analysis of DNA replication intermediates isolated from cells overexpressing Mge1p from high copy number plasmids. Equal signals from nonreplicating linear (“1N”) DNA for each strain indicate that equal amounts of DNA isolated from each strain were loaded on the gels.

**Fig. 4.** DNase I footprint analysis of ORC interactions with DNA in cells treated with adozelesin or MMS. A: Detection of the ORC-dependent hypersensitive site in the 2 µm origin of replication in cells arrested in mitosis with nocodazole. The first four lanes are a sequencing ladder indicating the position of the ARS consensus sequence
(ACS) in this origin. Arrow indicates position of ORC-dependent hypersensitive site (“ORC HS”) Slope indicates increasing digestion of chromatin. “0” indicates mock digestion with DNase I. “ND” indicates naked DNA control. B. Effect of MMS on DNA protein interactions at the 2 µm origin of replication in mitotic cells. Cells were treated with MMS at the indicated concentrations for 1 hour at 23°C. “Ch” is chromatin; “ND” is naked DNA. C. Effect of adozelesin on protein-DNA interactions at the 2 µm origin of replication in the presence of absence of expression of Mge1p. Drug-treated cells were exposed to 4 µM adozelesin for 1 hour at 23°C. Mge1 expression was repressed by raffinose (“+ raf”) or induced by galactose (“+ gal”). D. Quantitation of signals from the ORC-dependent hypersensitive site compared to adozelesin adducts at increasing concentrations of DNase I indicated by the number of “+” in 3 independent experiments.
Trabold et al., Figure 1
Trabold et al., Figure 2
Trabold et al., Figure 3
Activation of budding yeast replication origins and suppression of lethal DNA damage effects on origin function by ectopic expression of the co-chaperone protein Mge1

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