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MLH1 Deficiency Enhances Tumor Cell Sensitivity to Ganciclovir

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Abstract

Suicide gene therapy with herpes simplex virus thymidine kinase and ganciclovir is notable for producing multi-log cytotoxicity in a unique pattern of delayed cytotoxicity in S-phase. Because hydroxyurea, a ribonucleotide reductase inhibitor that activates mismatch repair, can increase sensitivity to ganciclovir, we evaluated the role of MLH1, an essential mismatch repair protein, in ganciclovir cytotoxicity. Using HCT116TK (HSV-TK-expressing) colon carcinoma cells that express or lack MLH1, cell survival studies demonstrated greater ganciclovir sensitivity in the MLH1 deficient cells, primarily at high concentrations. This could not be explained by differences in ganciclovir metabolism, as the less sensitive MLH1-expressing cells accumulated more ganciclovir triphosphate and incorporated more of the analog into DNA. siRNA suppression of MLH1 in U251 glioblastoma or SW480 colon carcinoma cells also enhanced sensitivity to high concentrations of ganciclovir. Studies in a panel of yeast deletion mutants confirmed the results with MLH1, and further suggested a role for homologous recombination repair and several cell cycle checkpoint proteins in ganciclovir cytotoxicity. These data suggest that MLH1 can prevent cytotoxicity with ganciclovir. Targeting mismatch repair-deficient tumors may increase efficacy of this suicide gene therapy approach to cancer treatment.

Keywords

ganciclovir; mismatch repair; MLH1

Introduction

In an effort to improve the selectivity of cancer chemotherapy, several suicide gene therapy strategies have been developed in which expression of a foreign gene in tumor cells activates a normally innocuous substrate to a cytotoxic metabolite.¹ One of the most widely investigated strategies employs transfer of the cDNA for the herpes simplex virus thymidine kinase (HSV-TK), and expression of the enzyme facilitates phosphorylation of the antiviral drug ganciclovir (GCV) to its 5'-monophosphate, GCVMP. After subsequent

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phosphorylation by endogenous kinases to its 5'-triphosphate (GCVTP), this metabolite competes with dGTP for incorporation into DNA which leads to cell death.¹ This approach has been successful in producing multi-log cell killing *in vitro* and strong tumor growth inhibition with some complete tumor regressions in animal models.²⁻⁴ These results have prompted clinical trials in patients with a variety of malignancies, and a combination therapy approach in prostate cancer. Clinical studies have demonstrated that HSV-TK/GCV therapy is well tolerated,⁵⁻⁷ with promising antitumor activity as part of a multimodality approach in prostate cancer.⁸

HSV-TK/GCV is notable for its ability to cause high cytotoxicity through a unique manner of delayed cell death distinct from other antimetabolites. Previously we demonstrated that GCV induced >3-logs more cell kill than other HSV-TK substrates, such as 1- β -D-arabinofuranosyl thymine (araT), despite the fact that more araT was incorporated into DNA than GCV.⁹ U251 glioblastoma cells were able to complete one cell division cycle after incubation with GCV for 24 hr. However, when they attempted to progress through the cell cycle for a second time, they were blocked in S phase where they remained until they died. In contrast, cells treated with araT accumulated in S phase and growth was inhibited for at least two days after drug removal, but subsequently surviving cells progressed through the cell cycle and cell number increased. This suggests that, with GCV treatment, an event occurring during this second round of DNA replication caused cells to arrest in S phase, resulting in cell death. Other reports demonstrated that, during a 48 – 72 hr continuous incubation in B16 murine melanoma cells, GCV induced a morphological change in cells due to the reorganization of components of the cytoskeleton¹⁰ and an accumulation of cells in S or G2/M.¹¹ In addition, GCV commonly induces an apoptotic cell death due to either a decline in Bcl-2 levels and activation of caspases¹², or through a CD95-dependent pathway.¹³

While these studies have documented changes in cell cycle progression and induction of apoptosis induced by GCV, the mechanism by which drug incorporation into DNA leads to these consequences is not known. Based on our previous data demonstrating that treatment with GCV arrested cells in S phase, we hypothesized that attempted repair of GCV in the template leads to cell death. Tomicic et al have implicated base excision repair in removal of GCVMP from DNA in CHO cells.¹⁴ Previously we reported that GCV cytotoxicity can be enhanced by the addition of hydroxyurea (HU), a ribonucleotide reductase inhibitor that produces an imbalance in dNTP pools, resulting in additive cytotoxicity in HSV-TK-expressing cells and synergistic cytotoxicity in non-HSV-TK-expressing bystander cells across a wide variety of solid tumor cell lines.¹⁵⁻¹⁷ Because HU causes an imbalance in dNTP pools which would lead to misincorporations and activation of the mismatch repair pathway (MMR),¹⁸⁻²⁰ this study aimed first to determine if MMR affects sensitivity to GCV. We have utilized HCT116 cells which are MMR deficient due to an inactivation of MLH1,²¹ along with the MMR-proficient cells in which the defect was corrected by expression of MLH1 cDNA.²² In addition, we further evaluated the role of MLH1 through siRNA-directed suppression of the protein in two different cell lines. Additional experiments in a yeast-based system investigated the role of proteins necessary for MMR and other DNA

repair pathways in GCV cytotoxicity, highlighting the importance of specific pathways which may be involved in sensing or repairing GCV-mediated DNA damage.

Materials and Methods

Cell Culture

HCT116 and SW480 human colon carcinoma and U251 human glioblastoma cell lines were maintained in Dulbecco's Modified Eagle medium, McCoy's, and RPMI (Invitrogen Life Technologies, Grand Island, NY); respectively. Media was supplemented with 2 mM L-glutamine (Fisher Scientific, Pittsburgh, PA) for all cell lines and 10% fetal bovine serum (Invitrogen) for HCT116 and SW480 and 10% bovine serum for U251 cells. All cells were maintained in exponential growth and kept in an atmosphere of 37°C and 5%CO₂.

Stable Gene-Expressing Cell Lines

HCT116 0-1, HCT116 1-2, SW480, and U251 cell lines were transduced with a retroviral vector encoding the herpes simplex virus type 1 thymidine kinase along with the neomycin resistance gene.³ Transgene expressing cells were selected with G418 and individual colonies were expanded and maintained in media containing G418 (Invitrogen). HSV-TK expression was confirmed by assaying lysates for phosphorylated GCV metabolites and immunoblotting for HSV-TK protein.

Clonogenic Cell Survival Assays

Exponentially growing cells were treated with GCV (Cytovene, Syntex, Palo Alto, CA) for 24 hr, trypsinized and diluted to approximately 100 viable cells per well in 6-well culture dishes. After 10–14 days, the cell colonies were fixed in methanol:acetic acid (3:1), stained with 0.4% crystal violet (Fisher Scientific), and visually counted. Cell survival is expressed as a fraction of the plating efficiency of control, non-drug treated cells. Each data point was plated in triplicate, and all assays were performed at least twice.

Cellular Nucleotide Analysis

After incubation with [8-³H]GCV (Moravek Biochemicals, Inc., Brea, CA), cells were harvested by trypsinization and extracted with 0.4 N perchloric acid and neutralized following drug exposure. The acid-insoluble pellets containing radiolabeled DNA were washed with 0.4 N perchloric acid and solubilized overnight in 1 N KOH. Incorporation of [8-³H]GCV into DNA was quantitated by liquid scintillation spectrometry. For analysis of cellular GCV triphosphate, fractions containing [8-³H]GCV triphosphate were collected and quantitated by liquid scintillation spectrometry based on the known specific activity of [8-³H]GCV.

Depletion of MLH1 with small interfering RNA

Cells were plated on 6 well plates at a density of 1.0×10^5 cells/ml and incubated for 24 hours. Cells were transfected with 100 nM siRNA directed to MLH1 or non-specific siRNA (Dharmacon, Lafayette, CO) and Lipofectamine 2000 (Invitrogen, Grand Island, NY). At 24 hours post-transfection, media was replaced. Cells were expanded at 48 hours post-

transfection and incubated for an additional 48 hours. Drug was added for 24 hours, and clonogenic cell survival assays were performed. Cell lysates were collected at time of drug addition for Western Blot analysis of MLH1 levels.

Immunoblot analysis

Whole-cell lysates were prepared in buffer [0.5 M Tris-HCl, 1.5 M NaCl, 2.5% deoxycholic acid, 10% NP40, 10 mM EDTA (pH 7.4)], with the addition of protease inhibitors (Complete Mini Protease Inhibitor Cocktail tablet, Roche, Indianapolis, IN). Proteins were separated by SDS-PAGE on 10% acrylamide gels and transferred onto Immobilon-P membrane (Millipore Corp., Bedford, MA). Blots were probed with MLH1 polyclonal rabbit (Santa Cruz Biotechnology, Santa Cruz, CA) or HSV-TK polyclonal rabbit antibodies and anti-rabbit horseradish peroxidase-linked antibodies. Proteins were visualized using an enhanced chemiluminescence detection system (Pierce, Rockford, IL).

Saccharomyces cerevisiae strains and expression constructs

The base yeast strain used in these experiments, YW929 (*MAT α* , *ade2::STE3-MET15*, *his3* 1, *leu2* 0, *met15* 0, *ura3* 0), was derived from the previously described suicide deletion strain YW798 by allowing the latter to undergo chromosome breakage and repair and selecting an *ade2* mutant product clone²³. HSV-TK and deoxycytidine kinase (dCK) expression constructs were created by amplifying the corresponding coding sequences with tailed primers so that the products could be ligated as a *Bam* HI-*Sal* I fragment into the previously described expression vector pTW300.²⁴ The product plasmids pTW382 and pTW383 express dCK and HSV-TK, respectively, from the strong constitutive *ADHI* promoter with a Myc epitope and His6 tag fused to the amino terminus. Chromosomal expression constructs were then made by amplifying the *ADHI*-Myc-His6-dCK/HSV-TK cassettes by PCR using primers with tails homologous to the yeast *CANI* gene. The fragments were transformed into YW929, canavanine-resistant *can1* colonies identified, and correct integration verified by PCR, α -Myc Western blot, and demonstration of drug-specific toxicity. The resulting yeast strains were YW967 (YW929 *can1* ::*ADHI*-dCK) and YW968 (YW929 *can1* ::*ADHI*-TK). Primer sequences are available on request.

Introduction of yeast gene deletion mutations was accomplished by a previously described mating strategy.²⁵ Briefly, YW968 was mated in array format to a previously described single-plate array of 96 DNA damage response gene deletion mutants.²⁵ Following selection of diploids and sporulation, recombinant haploids of the genotype *MAT α* , *ade2::STE3-MET15*, *can1* ::*ADHI*-TK, *his3* 1, *leu2* 0, *met15* 0, *ura3* 0, xxx ::kanMX4 (where xxx refers to the various deleted genes) were identified by their growth as red (i.e. *ade2*) colonies on plates selective for methionine and containing canavanine and G418.

Measurement of GCV sensitivity in *Saccharomyces cerevisiae*

Overnight cultures were diluted 50-fold in synthetically defined media with glucose as the carbon source²⁴ and allowed to grow for 5 hr shaking at 280 rpm at 30° C. Cultures were then diluted to a calculated OD₆₀₀ = 0.0005 in the same media containing varying concentrations of GCV. Growth was continued until the OD₆₀₀ of the untreated control reached 0.5 \pm 0.15 (~10 doublings). The OD₆₀₀ of all cultures was then determined. Values

are expressed as a fraction of the optical density of the corresponding untreated control sample.

Complementation of mutant *Saccharomyces cerevisiae* strains

PCR primers were designed to amplify the gene of interest (coding sequence plus 1000 bp upstream of the start codon) from wild-type yeast genomic DNA. Primers included tail regions (forward: 5'-TGGCGGCCGCTCTAGAAGTAGTGGATCCCC, reverse: 5'-GATAAGCTTGATATCGAATT CCTGCAGCCC) to allow gap repair of *Sma* I-digested vector pRS316 (*URA3*, CEN/ARS). Digested plasmid and PCR products were co-transformed into yeast strains containing the corresponding gene deletions to generate recombinant plasmids. Colonies were picked and tested for sensitivity to GCV as described above.

Results

To explore the role of MMR in the cytotoxicity of GCV, these studies utilized the HCT116 0-1 cells, which are MMR deficient due to a truncated essential protein for MMR (MLH1),²¹ and HCT116 1-2 cells which stably express MLH1 from its full length cDNA and are MMR proficient. To facilitate phosphorylation of GCV to its active 5'-triphosphate, stably expressing HSV-TK clonal sublines were generated for both the MMR-deficient HCT116 0-1 and the MMR-proficient HCT116 1-2 cell lines. As illustrated in Figure 1, both of the HCT116 0-1tk clones (MMR deficient) were more sensitive to GCV than any of the HCT116 1-2tk clones (MMR proficient), especially at high GCV concentrations. One clonal subline from each cell line was chosen based on similar growth rates and sensitivity to GCV. Cytotoxicity of GCV in these two clonal sublines was similar at concentrations of 1 μ M or less (% control survival at 1 μ M = $3 \pm 0.5\%$ and $5.2 \pm 1.4\%$); however, at 10 μ M GCV, greater than one log more cell kill was observed in the MMR-deficient 0-1tk cells compared to the MMR-proficient 1-2tk cells (percent survival = $0.05 \pm 0.03\%$ and $0.72 \pm 0.2\%$, respectively; $p = 0.0046$).

To determine whether the difference in cytotoxicity could be explained by differential metabolism of GCV in the two cell lines, we measured accumulation of GCVTP and its incorporation into DNA. In both cell lines, there was an increase in GCVTP during drug incubation, and a subsequent decrease in GCVTP levels following drug washout (Figure 2A). The HCT116 1-2tk clone accumulated approximately 3 times more GCVTP than the HCT116 0-1tk clone following treatment with 1 μ M GCV (55.9 ± 3.9 pmol GCVTP/ 10^6 cells and 17.8 ± 1.4 pmol GCVTP/ 10^6 cells, respectively). There was an increase in the amount of GCVTP incorporated into DNA during drug incubation and for 6 to 8 hours following drug removal. The HCT116 1-2tk cells incorporated approximately two-fold more GCVMP into DNA than the 0-1tk cells (5.3 ± 0.3 pmol GCVMP/ 10^6 cells, 2.4 ± 0.01 pmol GCVMP/ 10^6 cells, respectively), consistent with the higher pool of GCVTP (Figure 2B). GCVMP was well-retained in DNA in both sublines for at least 48 hr after drug washout. The slight decrease detected in HCT116 0-1tk cells was accounted for by an increase in cell number (data not shown). Interestingly, 1 μ M GCV was equitoxic in these two clones, despite the fact that there was twice as much GCVMP in the DNA of the 1-2tk clone.

Similar results were obtained at 10 μM GCV in which the HCT116 1-2tk cells accumulated up to 4 times more GCVTP and up to 2 times more GCVMP in DNA compared to the HCT116 0-1tk cells (data not shown). Western blot analysis demonstrated that HCT116 1-2tk clone expressed 2.5-fold more HSV-TK than the HCT116 0-1tk clone, which accounts for the higher GCVTP accumulation and GCVMP incorporation into DNA observed in the HCT116 1-2tk clone (Figure 1B). Thus, reduced metabolism does not account for the lower sensitivity to GCV of the HCT116 1-2tk cells.

Because differential expression of HSV-TK in the two clones resulted in different levels of GCVTP, the cytotoxicity of GCV was tested in the parental (non-HSV-TK expressing) HCT116 cell lines. If cells that do not express HSV-TK are treated with high concentrations of GCV, the drug can be phosphorylated by cellular enzymes,²⁶ and it was reasonable to assume that phosphorylation would be equivalent in the parental cell lines. Similar to the results in the HSV-TK-expressing cells, the MMR-deficient HCT116 0-1 cells were more sensitive to GCV than the MMR-proficient HCT116 1-2 cells ($\text{IC}_{50} = 120 \pm 5.8 \mu\text{M}$ and $477 \pm 23.3 \mu\text{M}$, respectively; $p=0.0001$) (Figure 1C), with >1-log difference in survival at GCV concentrations 300 μM , suggesting that the higher sensitivity of the HSV-TK-expressing HCT116 0-1 cells was due to MMR deficiency.

Because MMR deficiency produces a mutator phenotype which may have affected sensitivity to GCV in the HCT116 0-1 cells, siRNA was used to suppress MLH1 expression in two other cell lines, U251tk human glioblastoma and SW480tk human colon carcinoma, both of which stably expressed HSV-TK and are considered MMR proficient.²⁷ Transfection with MLH1 siRNA decreased MLH1 expression to 11.8 and 1.3% of control in the SW480tk and U251tk cells, respectively (Figures 3A and C). Suppression of MLH1 expression increased the sensitivity of both cell lines to GCV, primarily at drug concentrations that produced high cytotoxicity (>90% cell killing) (Figures 3B and 3.5D). Although there was not a complete deficiency of MLH1 in these studies, sensitivity to GCV was increased significantly, observed by a decrease in the IC_{99} for GCV from $6.25 \pm 0.92 \mu\text{M}$ (nonspecific siRNA) to $1.66 \pm 0.11 \mu\text{M}$ (MLH1 siRNA) in SW480tk cells ($p = 0.02$) and from $0.7 \pm 0.02 \mu\text{M}$ (nonspecific siRNA) to $0.44 \pm 0.07 \mu\text{M}$ (MLH1 siRNA) in U251tk cells ($p = 0.02$). Depletion of MLH1 in SW480tk cells decreased survival with 10 μM GCV by more than 90-fold (survival = 1.13% and .012% with nonspecific or MLH1 siRNA, respectively). Similarly, in U251tk cells depletion of MLH1 decreased survival with 0.5 μM GCV by nearly 40-fold from 4.2% to 0.1% with nonspecific or MLH1 siRNA, respectively.

In order to evaluate other DNA repair pathways that participate in repair of GCV-induced lesions, a *Saccharomyces cerevisiae*-based genetic screen was utilized to discover DNA damage response mutants with increased sensitivity to GCV. HSV-TK was placed under control of the strong constitutive yeast *ADHI* promoter and integrated into yeast chromosome V to stably express the gene. GCV conferred dose-dependent toxicity only in HSV-TK-expressing yeast and not in control yeast or those expressing deoxycytidine kinase (dCK) (Figure 4). Note that higher concentrations of GCV were required to affect growth of HSV-TK-expressing yeast compared to mammalian cells, which is typical in yeast likely due to poor transport properties for many drugs²⁸ and the high capacity of yeast for DNA repair, especially homologous recombination repair.²⁹

HSV-TK expression was next introduced into a panel of 96 DNA damage response yeast deletion mutants and the resulting strains screened for sensitivity to GCV. Table 1 indicates that MMR mutants exhibited a low to moderate increase in sensitivity to GCV at the concentrations tested. Mutants deficient in the MMR genes *MLH1* or *MSH2* were as sensitive as controls at a moderate concentration of GCV (0.3 mM) but exhibited significantly increased sensitivity at 5 mM (% control density = 62.4 ± 1.9 for the WT-HSV-TK strain and 46.2 ± 1.1 ($p < 0.01$) and 50.1 ± 4.0 ($p < 0.05$) for *mlh1* and *msh2* yeast, respectively). Yeast with deletions in homologous recombination repair (HRR) or cell cycle checkpoint genes exhibited high sensitivity to GCV. Deletion mutants for the endonucleases *MUS81* or *MMS4* also showed high sensitivity to GCV, which may be due to their putative role in HRR.³⁰ In addition, the increased sensitivity of the *asf1* mutant may be due to its role as a histone chaperone protein which may implicate it in HRR or other DNA repair pathways.³¹

In order to verify that the deleted genes were in fact responsible for the increased sensitivity to GCV, representative mutant strains (RecQ helicase, *sgs1*; MRX subunit involved in double strand break repair, *rad50*; homologous recombination protein, *rad52*; and checkpoint protein, *dun1*) with moderate to high GCV sensitivity were complemented with the corresponding wild-type gene in a plasmid. In all cases, complementation resulted in decreased sensitivity to GCV, similar to wild-type levels (Figure 5A–D) as expected, thus verifying that the gene deletion was responsible for the increased drug sensitivity.

Discussion

Previously we have demonstrated a strong S-phase block associated with GCV cytotoxicity, suggesting that GCV produced irreparable DNA damage.⁹ However, the type of damage and the repair pathways that may be involved in repairing GCV-induced DNA damage have not been identified. Here we have evaluated the role of MLH1, a protein required for MMR, in the cytotoxicity of GCV using three different human cell lines of varying sensitivity to GCV. Additional pathways that may be involved in GCV cytotoxicity were identified using a yeast deletion mutant assay. The results demonstrated that, at high concentrations of GCV, human or yeast cells that expressed MLH1 are less sensitive to GCV induced cytotoxicity. The yeast assay also implicated HRR in GCV cytotoxicity. These results suggest that GCV induces specific lesions that can be repaired by MMR or HRR, and impairment of these pathways leads to increased cytotoxicity.

The increase in cytotoxicity in HCT116 0–1 cells (MMR deficient due to lack of functional MLH1) compared to HCT116 1–2 cells (MMR proficient due to expression of functional MLH1) was not due to an increase in GCVTP or its incorporation into DNA, since the HCT116 1–2 cells actually accumulated more GCVMP in DNA. Further evidence for a direct role of MLH1 in cytotoxicity of GCV was demonstrated by the results in the U251 and SW480 cells using MLH1-specific siRNA, as well as the yeast assay which all demonstrated increased cytotoxicity of GCV in cells deficient in MLH1, primarily at high drug concentrations. Using siRNA to suppress MLH1 was important because it controlled for any differences the matched HCT116 cell lines may have accumulated, after many years of being cultured separately, that could affect GCV sensitivity. While the siRNA did not

result in a complete reduction of MLH1, these cells still displayed an increase in sensitivity to GCV. Furthermore, the siRNA studies demonstrated that two different cell lines which differed in inherent sensitivity to GCV both exhibited increased sensitivity at IC_{90} for GCV when MLH1 expression was decreased.

There are several possibilities for the mechanism by which MLH1 deficiency enhances GCV cytotoxicity. Although MLH1 deficiency did not enhance incorporation of GCVMP into DNA, it may have allowed the persistence of other DNA lesions, such as mismatched nucleotides. MLH1 is a required protein for MMR, and therefore if high concentrations of GCV induced mismatches during DNA replication, deficiency of MLH1 would lead to more errors which may enhance cytotoxicity. Preliminary data indicate that GCV can induce mismatches in DNA but only at high concentrations,³² thus lending support to this hypothesis. Alternatively, MLH1 may protect cells from GCV-induced damage through downstream signaling, since MLH1 is known to participate in a variety of other pathways such as base excision repair, cell cycle checkpoints, and apoptosis.^{33–35} These hypotheses are currently under investigation.

Previously we have demonstrated that HU enhances cell killing with GCV,^{15–17} and we suggested that this occurred through the increased incorporation of GCVMP into DNA due to the HU-mediated decrease in dGTP. Alternatively, since HU-mediated dNTP pool imbalances activate MMR, it was also possible that HU enhanced cytotoxicity of GCV through activation of MMR which might increase incorporation of GCVMP into DNA as HU-induced mismatches were repaired, as suggested previously for the increased sensitivity of gemcitabine in MMR-proficient cells.³⁶ However, direct evaluation in MMR-proficient and deficient cell lines here demonstrated that deletion or suppression of the required MMR protein, MLH1, actually enhanced cytotoxicity at GCV concentrations IC_{90} . In contrast, most of the GCV/HU combination studies demonstrated strong synergistic cytotoxicity at concentrations of GCV $<IC_{90}$. Taken together, these data support the notion that the combination of GCV and HU elicit synergy by increasing GCVMP in DNA rather than through activation of MMR.

We extended the results with MMR to screen a panel of yeast strains containing deletions in various DNA damage and repair genes to evaluate other pathways which may play a role at lower concentrations of GCV. DNA damage repair pathways and checkpoints in *S. cerevisiae* are conserved with those in humans.³⁷ The yeast system allows for a rapid screen of many different mutants, a process which would be very difficult to conduct in mammalian cells due to the amount of time required to develop and test a large number of deletion mutants. Experiments in yeast have correctly predicted effects in human cells with other drugs as well. Previous work from the Wilson laboratory and others identified the major Tdp1-dependent pathway for resolution of aberrant topoisomerase complexes in yeast,^{25,38} findings which were subsequently confirmed in human cells.³⁹ These data support the use of the yeast assay to discover other DNA damage response pathways that affect sensitivity to GCV.

Results from the yeast assay supported the results in the human cell lines, in which deletions in MMR genes *MLH1* or *MSH2* enhanced sensitivity to GCV primarily at high

concentrations of GCV. The yeast assay also demonstrated that deletion of genes involved in HRR caused a significant increase in sensitivity to GCV. Deletion of *SGS1*, a helicase involved in HRR, and *MMS4* or *MUS81*, which function together to cleave sites of stalled replication forks and lead to initiation of HRR also resulted in increased sensitivity to GCV. Interestingly, deletion of genes involved in postreplication repair and base excision repair did not confer sensitivity to GCV, suggesting that these pathways are not involved in protecting from GCV-mediated cytotoxicity. Although a study in Chinese hamster ovary cells suggested that base excision repair is involved in protection of cells to GCV,¹⁴ we have not observed excision of GCVMP from DNA in a variety of human cell lines.^{3,9,26}

There are several mechanisms through which HRR could impact GCV cytotoxicity. Thust et al have demonstrated that GCV induces sister chromatid exchanges, which usually arise from HRR, during the second S phase after GCV exposure.^{40,41} In addition, HRR is required to restart stalled replication forks,⁴² and we have also shown that, at concentrations IC_{50} , GCV slows replication which likely is due to stalled replication forks.¹⁶ Thus, it will be important to determine the precise role of HRR in GCV cytotoxicity.

Deletion of the gene for *DUNI*, a regulator of ribonucleotide reductase^{43,44} which produces dNTPs for DNA replication and repair, also enhanced the sensitivity of yeast significantly to GCV. The absence of this protein would result in lower dNTP pools, impairing DNA replication and repair following GCV-induced DNA damage. In addition, lowered dNTP pools would likely result in an increase in GCVTP incorporation into DNA by decreasing the availability of its competitor, dGTP, another mechanism for increased cytotoxicity. Although a human homolog of *DUNI* has not been discovered, we have previously demonstrated a role for ribonucleotide reductase since its inhibition enhanced GCV cytotoxicity.^{15,16,45}

The yeast assay also implicated several cell cycle checkpoint proteins in enhancing GCV cytotoxicity. Ddc1, Mec3, and Rad17 are yeast homologs of the 9-1-1 complex that responds to DNA damage in mammalian cells, and Rad24 loads the complex onto damaged DNA.⁴⁶ This complex is involved in facilitating activation of Chk1, resulting in checkpoint activation and cell cycle arrest to allow time for cells to repair DNA damage. Thus, absence of these critical proteins may decrease repair of GCV-induced DNA damage. These data will need to be confirmed in human cells.

These results suggest a variety of mechanisms to improve therapy with HSV-TK/GCV. The data suggest that HSV-TK/GCV may be more effective in MLH1-deficient tumors, which is a common finding in many different human cancers.⁴⁷ In support of this idea, the most promising results in clinical trials of this therapy have been in prostate cancer,⁴⁸ a tumor type in which a significant percentage show loss of at least one MMR protein.⁴⁷ Although the increased sensitivity of MLH1 deficient cells occurred at high concentrations of GCV (0.5–10 μ M), these are well within the clinically relevant range as GCV typically achieves plasma concentrations of 10–30 μ M in patients.^{49,50} If data from the yeast screen were confirmed in human tumor cells, it may be advantageous to target HRR in conjunction with HSV-TK/GCV. Furthermore, tumors defective in cell cycle checkpoint proteins would be expected to respond better to GCV. Since normal tissues are generally proficient in these

pathways, targeting MMR and checkpoint defective tumors would improve selectivity of this therapy. Further exploration of the role of these pathways in the antitumor activity of HSV-TK/GCV using *in vivo* models will help to optimize therapeutic efficacy of this approach.

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Abbreviations

araT	1- β -D-arabinofuranosyl thymine
dCK	deoxycytidine kinase
GCV	ganciclovir
GCVMP	ganciclovir monophosphate
GCVTP	ganciclovir 5'-triphosphate
HSV-TK	herpes simplex virus thymidine kinase
HRR	homologous recombination repair
HU	hydroxyurea
MMR	mismatch repair

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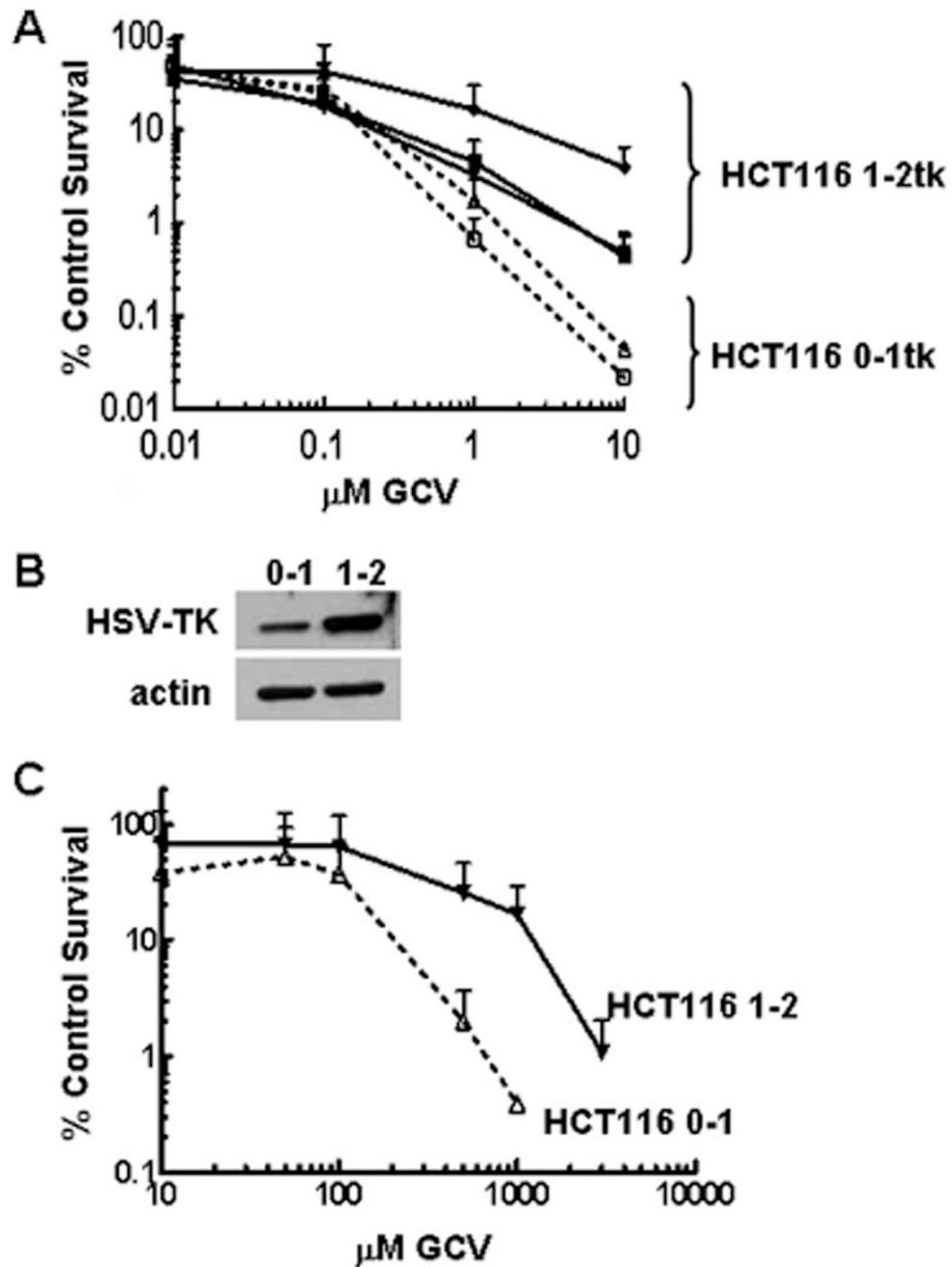


Figure 1. Sensitivity of HCT116 cells to GCV. Exponentially growing HCT116 0-1 (dashed line) and 1-2 cells (solid line) (A) stably expressing HSV-TK or (C) non-HSV-TK expressing cells were exposed to increasing concentrations of GCV for 24 hours. Clonogenic cell survival was determined and expressed as a fraction of plating efficiency for untreated cells. Points represent a mean of triplicate samples, bars represent standard error. Cell lines were chosen for use in subsequent experiments: HCT116 1-2tk (\blacktriangle) and HCT116 0-1tk (\square). (B) Whole

cell lysates were analyzed by Western blotting for HSV-TK expression. Expression of actin was used as a loading control.

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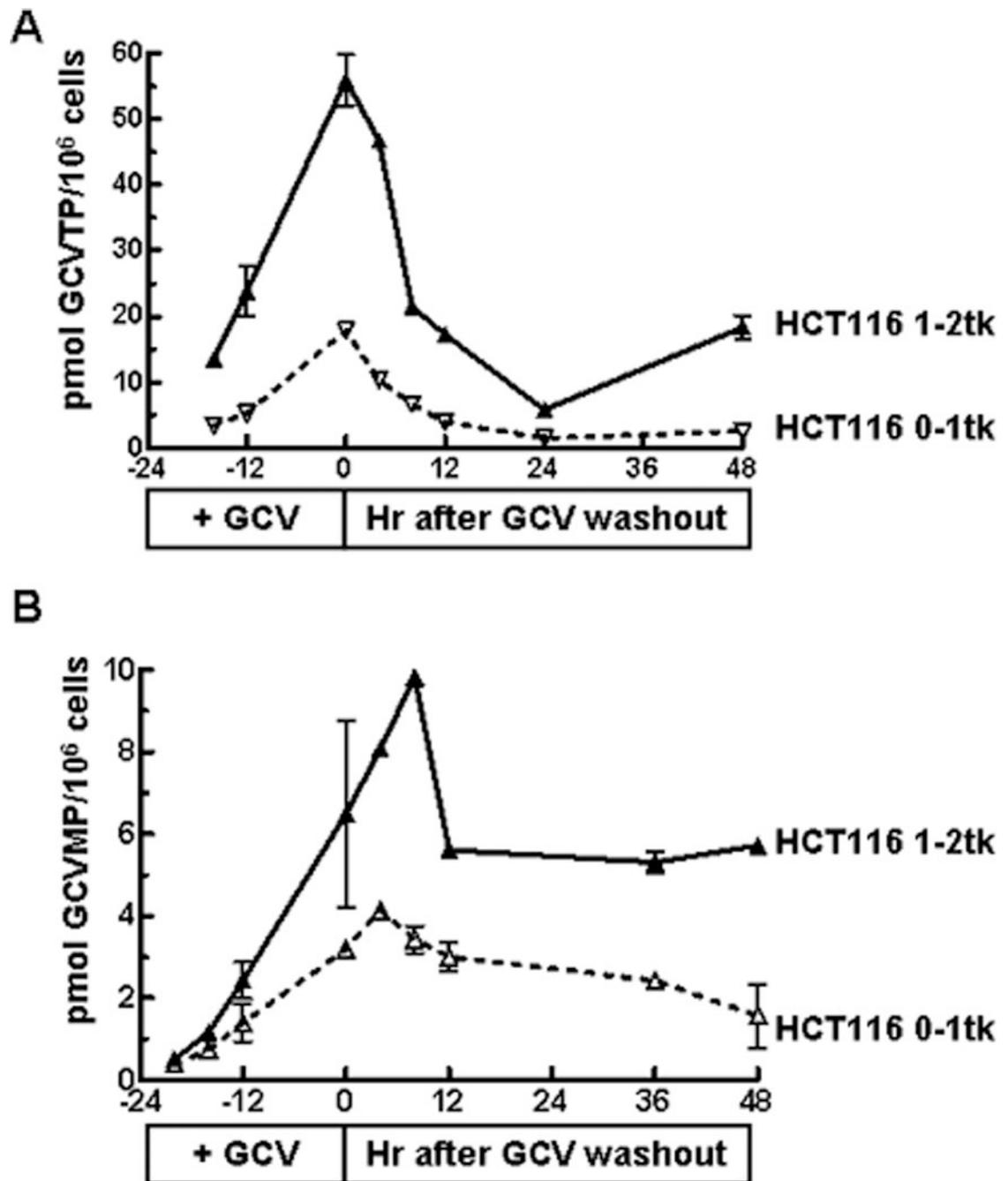
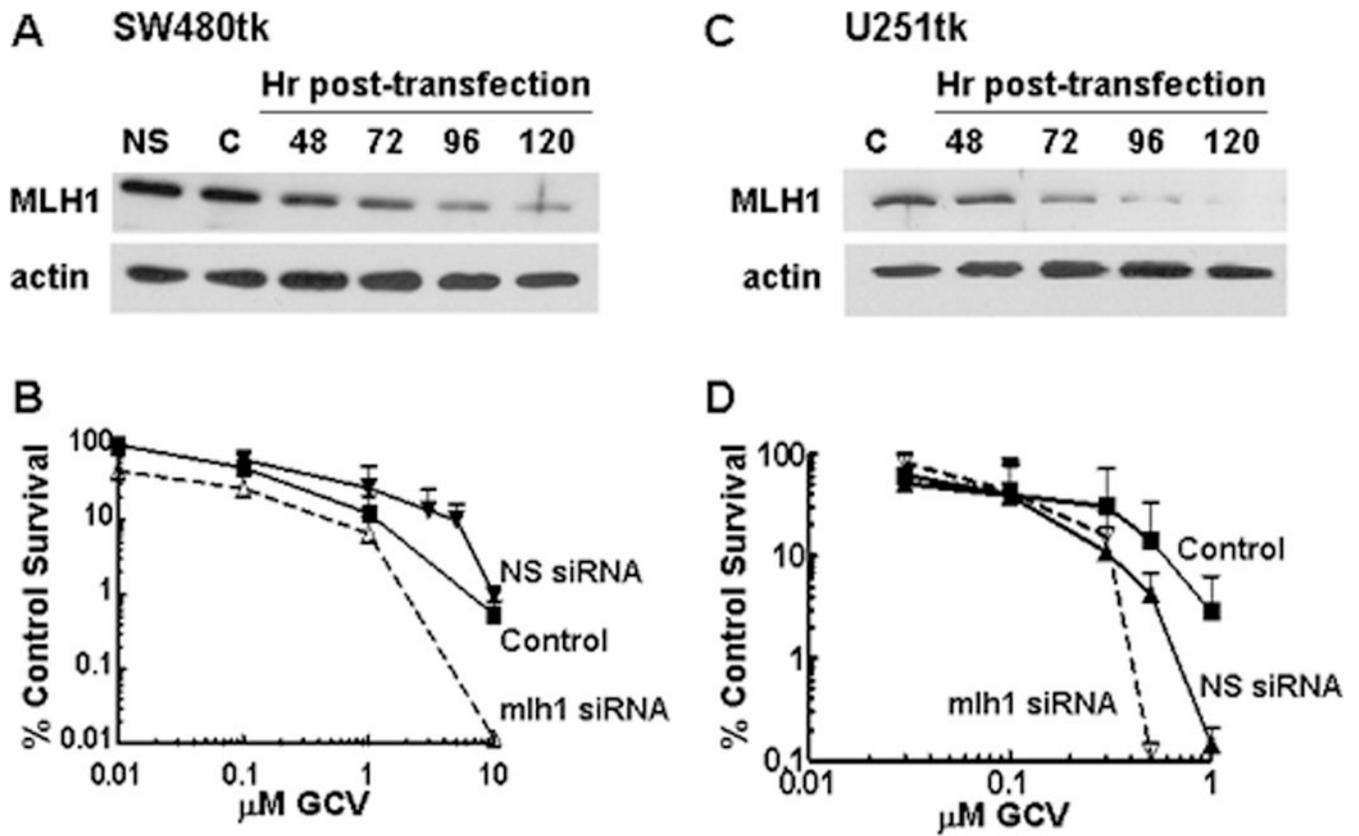


Figure 2. Accumulation of GCVTP and its incorporation into DNA. HCT116 cells were incubated with [³H]GCV for 24 hr followed by drug washout. (A), GCVTP was measured by HPLC, and (B), GCVMP in DNA was measured in the acid-insoluble cell fraction in HCT116 0-1tk (dashed line) and HCT116 1-2tk (solid line). Points represent the mean of at least triplicate samples, bars represent standard error.

**Figure 3.**

Depletion of MLH1 results in increased sensitivity to GCV. (A and B), SW480tk cells; (C and D), U251tk cells. (A and C), Whole cell lysates were analyzed by Western blotting for MLH1 expression following siRNA transfection. Expression of actin was used as a loading control. (B and D), Sensitivity of cells treated with siRNA directed to MLH1 (gray line), non-specific siRNA (dashed line), or no siRNA (solid line) was determined following exposure to increasing concentrations of GCV. Points represent the mean of triplicate experiments, bars represent standard error.

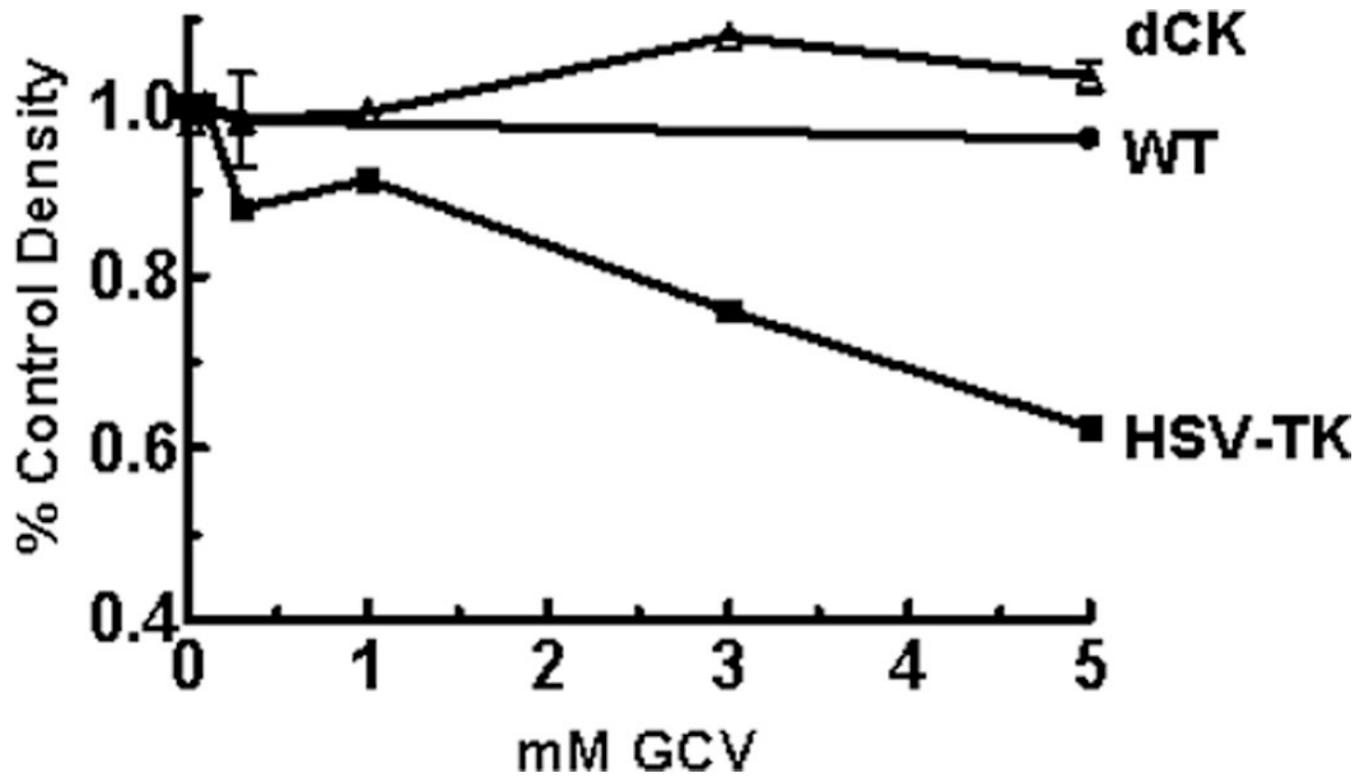


Figure 4. Sensitivity of *S. cerevisiae* to GCV. Exponentially growing liquid cultures of *S. cerevisiae* strains expressing HSV-TK, dCK, or no exogenous enzyme were treated with increasing concentrations of GCV. Cell density was determined and expressed as a fraction of the density of untreated control cultures. Points represent the mean of triplicate experiments, bars represent standard error.

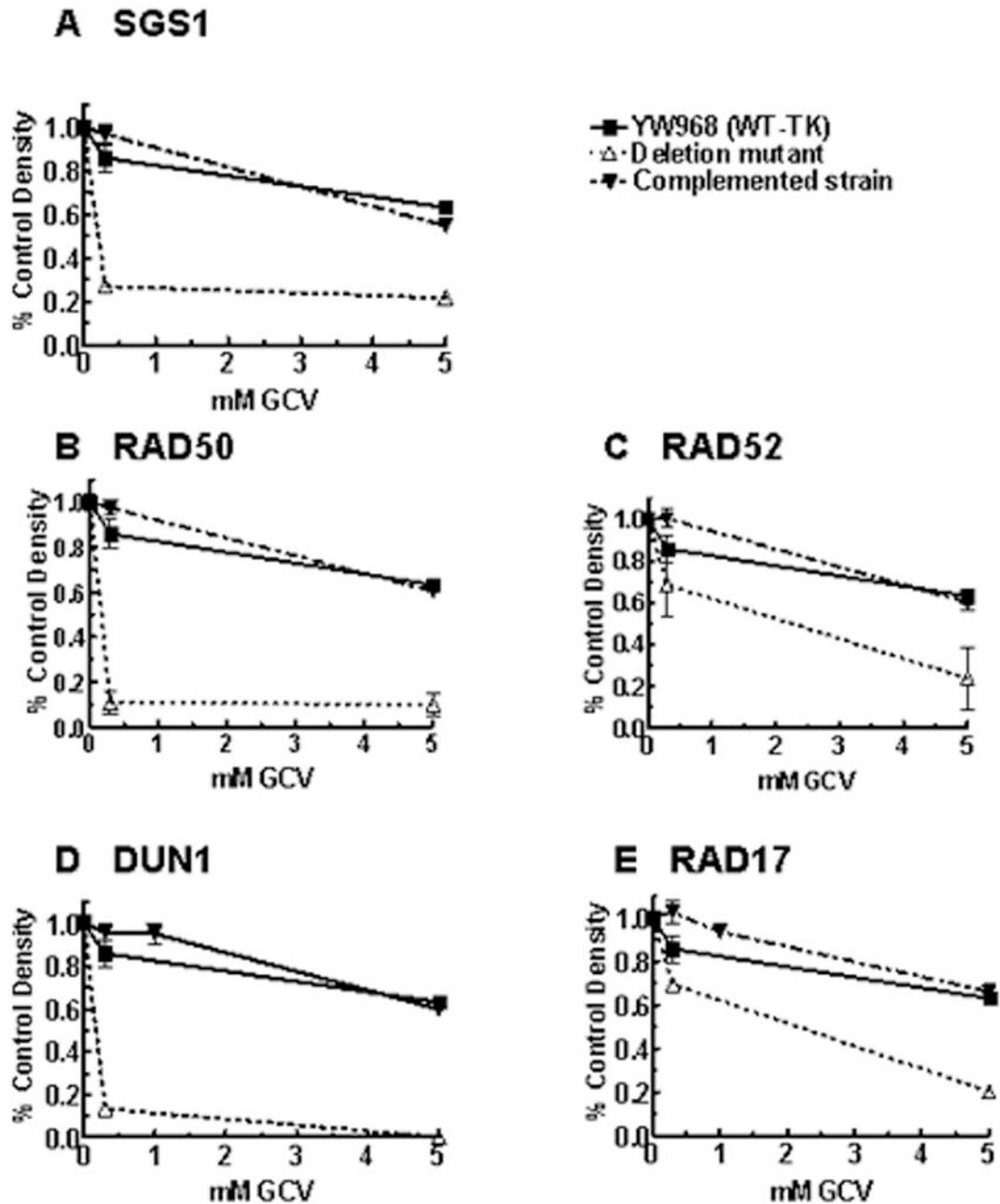


Figure 5. Sensitivity of *S. cerevisiae* deletion mutants to GCV. Exponentially growing liquid cultures of *S. cerevisiae* strains expressing HSV-TK and bearing specific gene deletion mutations [(A) *sgs2*, (B) *rad50*, (C) *rad52*, (D) *dun1*] and their complemented counterparts were treated with increasing concentrations of GCV and cell density determined as in Figure 4. Solid lines and squares indicate wild-type yeast, dotted lines with open triangles indicate specific deletion mutants, and dashed and dotted lines with closed triangles indicate

complemented strains. Points represent the mean of triplicate experiments, bars represent standard error.

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Table 1Sensitivity of Yeast Strains to Ganciclovir^a

Function	GeneName	GCV
RecQ/Topo III helicase	TOP3	++
	SGS1	+++
	HPR5	++
MRX complex	RAD50	+++
homologous recombination	RAD51	+++
	RAD55	+++
	RAD57	+++
	RAD54	+
	RAD52	++
	RAD59	+++
	RDH54	-
	checkpoint	DUN1
RAD24		++
DDC1		++
MEC3		+++
RAD9		+
RAD17		++
TEL1		-
endonuclease	MUS81	+++
	MMS4	+++
chromatin	CHD1	-
	ASF1	+++
mismatch repair	MLH1	+
	MSH1	++
	MSH2	+
	PMS1	+
post-replication repair	RAD6	-
	RAD18	-
	RAD5	+
replication	CTF4	-
	POL32	+
	RAD27	-
	DPB3	-
base excision repair	APN1	-

^aNOTE: +++ indicates significant increase in sensitivity to 0.3 mM GCV (50% control density), ++ indicates significant increase in sensitivity to 5 mM GCV (25% control density), + indicates significant increase in sensitivity to 5 mM GCV (50% control density), and – indicates no difference from control. The results were first determined by analysis of the array in 96 well plates and validated in triplicate in 2 ml liquid cultures.

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