

Replication Fork Collapse and Genome Instability in a Deoxycytidylate Deaminase Mutant

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Ribonucleotide reductase (RNR) and deoxycytidylate deaminase (dCMP deaminase) are pivotal allosteric enzymes required to maintain adequate pools of deoxyribonucleoside triphosphates (dNTPs) for DNA synthesis and repair. Whereas RNR inhibition slows DNA replication and activates checkpoint responses, the effect of dCMP deaminase deficiency is largely unknown. Here, we report that deleting the *Schizosaccharomyces pombe dcd1⁺* dCMP deaminase gene (SPBC2G2.13c) increases dCTP ~30-fold and decreases dTTP ~4-fold. In contrast to the robust growth of a *Saccharomyces cerevisiae dcd1Δ* mutant, fission yeast *dcd1Δ* cells delay cell cycle progression in early S phase and are sensitive to multiple DNA-damaging agents, indicating impaired DNA replication and repair. DNA content profiling of *dcd1Δ* cells differs from an RNR-deficient mutant. Dcd1 deficiency activates genome integrity checkpoints enforced by Rad3 (ATR), Cds1 (Chk2), and Chk1 and creates critical requirements for proteins involved in recovery from replication fork collapse, including the γ H2AX-binding protein Brc1 and Mus81 Holliday junction resolvase. These effects correlate with increased nuclear foci of the single-stranded DNA binding protein RPA and the homologous recombination repair protein Rad52. Moreover, Brc1 suppresses spontaneous mutagenesis in *dcd1Δ* cells. We propose that replication forks stall and collapse in *dcd1Δ* cells, burdening DNA damage and checkpoint responses to maintain genome integrity.

The accurate duplication of a eukaryotic genome demands abundant supplies of deoxyribonucleoside triphosphates (dNTPs), which are the building blocks of DNA. Much of the burden for providing both ample and balanced pools of dNTPs falls to two allosteric enzymes: ribonucleotide reductase (RNR) and deoxycytidylate deaminase (dCMP deaminase) (22, 31, 40). RNR plays essential roles in the *de novo* biosynthesis of all four dNTPs required for DNA synthesis, while dCMP deaminase is specifically involved in the production of dTTP (Fig. 1). Whereas the physiological consequences of RNR defects have been investigated in great detail (9, 15, 47), the effects of dCMP deaminase deficiency are much less well understood, despite its presumptive key roles in efficient genome duplication and in influencing the outcomes of nucleoside-based antitumor and antiviral therapies (24, 25, 34).

Investigations of dCMP deaminase in a genetically tractable organism have been carried out with the budding yeast *Saccharomyces cerevisiae* (29, 38). As predicted, null mutations of the *DCD1* dCMP deaminase gene significantly increase dCTP and decrease dTTP pools, resulting in an ~125-fold increase in the dCTP/dTTP ratio. Surprisingly, these dNTP pool imbalances do not reduce growth rates or have other obvious effects but rather modestly increase mutagenesis rates (29). In contrast, chemical or mutational inhibition of RNR slows DNA replication and activates checkpoint responses. Thus, RNR and dCMP deaminase deficiencies have quite different effects in *Saccharomyces cerevisiae*.

The fission yeast *Schizosaccharomyces pombe* has networks of genome protection mechanisms, ranging from highly specialized lesion repair enzymes to checkpoint mechanisms that coordinate multiple DNA damage responses (60). These include master checkpoint regulators such as the protein kinase Rad3 (ATR in mammals, Mec1 in *S. cerevisiae*). Rad3 phosphorylates many substrates, including the C terminus of histone H2A in chromatin flanking DNA lesions (45). Phosphohistone H2A (here γ H2A),

which is analogous to γ H2AX in mammals (6), provides a multi-kilobase recruitment platform for genome protection factors, notably Mdc1 in mammals and Crb2 in fission yeast (14, 27, 55, 59). Recently, we discovered that γ H2A also recruits Brc1, a protein with six BRCT domains, via docking of the phosphorylated C terminus of γ H2A with the C-terminal pair of BRCT domains in Brc1 (65). This interaction is conserved in *S. cerevisiae* Rtt107 and mammalian PTIP, which are also 6-BRCT domain proteins (36, 67). Brc1 is important for survival of collapsed replication forks (2). Here, we report that Brc1 and other genome protection proteins are critical for survival in the absence of dCMP deaminase, revealing that unbalanced dNTP pools in the dCMP deaminase mutant profoundly affect DNA replication and repair.

MATERIALS AND METHODS

Strains and genetic methods. Strains used in this study were made using standard techniques (17) and are listed in Table 1. The complete deletion of the *dcd1⁺* open reading frame was made using pFA6a-KanMX6 as the template and the primers Dcd1 Forward (5'-TGGGG AGATAATCAAACCTGAAATACGTTTCTTAAGCTTAAGTAGCTTTGT TTGATTTATTCAGCTGAAGCTTCGTACGCT-3') and Dcd1 Reverse (5'-ATAAAGATATTAATAATCGTATTTTCTGCAATCAAAAAGAGCG GTAATCTAAAAAGTAAGTCTAGTGGATCTGATATCATC-3'). The PCR product was transformed into the wild-type strain (*h⁻ leu1-32*) cells by the lithium acetate protocol (43), and disruption was verified by PCR.

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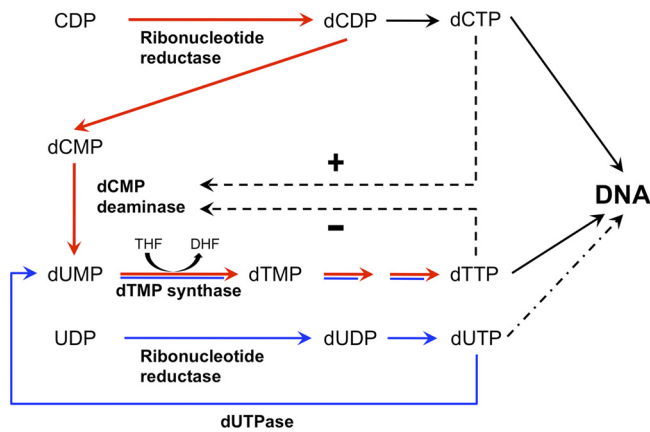


FIG 1 Simplified scheme of *de novo* dTMP synthesis. *De novo* synthesis of dCDP is catalyzed by ribonucleotide reductase. Conversion of dCMP to dUMP is catalyzed by dCMP deaminase, which is positively regulated by dCTP (+) and inhibited by dTTP (-). Red arrows indicate the major dTTP synthesis pathway via dCMP deaminase, and blue arrows indicate the minor dTTP synthesis pathway via dUTPase.

Tetrad analysis was performed to construct double mutants and verified by PCR.

Survival assays. For DNA damage sensitivity assays, cells were grown in YES (yeast extract, glucose, and supplements) medium to log phase, plated as 10-fold serial dilutions, and treated with indicated amounts of hydroxyurea (HU), camptothecin (CPT), methyl methanesulfonate (MMS), 4-nitroquinoline N-oxide (4-NQO), and bleomycin. For UV treatment, cells were serially diluted onto YES plates and irradiated using a Stratagene UV source. Cell survival was determined after 3 to 4 days at 30°C.

Determination of dNTP and NTP pools. At a density of 0.4×10^7 to 0.5×10^7 cells/ml, $\sim 3.70 \times 10^8$ (as determined by the optical density at 600 nm [OD₆₀₀]) cells were harvested by filtration through 25-mm white AAWP nitrocellulose filters (0.8 mm; Millipore AB, Solna, Sweden). To determine the exact number of cells, the cells were also counted in a hemocytometer. The filters were immersed in 700 µl of ice-cold extraction solution (12% [wt/vol] trichloroacetic acid, 15 mM MgCl₂) in Eppendorf tubes. The following steps were carried out at 4°C. The tubes were vortexed for 30 s, incubated for 15 min, and vortexed again for 30 s. The filters were removed, and the 700-µl supernatants were collected after centrifugation at 20,000 × g for 1 min and added to 800 µl of ice-cold Freon-trioctylamine mixture (10 ml of 99% pure Freon [1,1,2-trichloro-

TABLE 1 *S. pombe* strains used in this study

Strain	Genotype	Source
AS237	<i>h⁻ leu1-32</i>	This study
PR110	<i>h⁺ leu1-32 ura4-D18</i>	Laboratory stock
AS137	<i>h⁻ leu1-32 brc1::hphMX6</i>	This study
AS304	<i>h⁻ leu1-32 dcd1::kanMX6</i>	This study
AS351	<i>h⁻ leu1-32 ura4-D18 dcd1::kanMX6</i>	This study
AS281	<i>h⁻ leu1-32 dcd1::kanMX6 brc1::hphMX6</i>	This study
KS1452	<i>h⁻ leu1-32 ura4-D18 ade6-704 chk1Δ::ura4⁺</i>	S. Forsburg
AS517	<i>h⁺ leu1-32 ura4-D18 ade6-704 dcd1::kanMX6 chk1Δ::ura4⁺</i>	This study
NB2117	<i>h⁻ leu1-32 ura4-D18 cds1Δ::ura4⁺</i>	Laboratory stock
KT2752	<i>h⁻ leu1-32 ura4-D18 cds1-T8A</i>	Laboratory stock
AS809	<i>h⁺ leu1-32 ura4-D18 dcd1::kanMX6 cds1-T8A</i>	This study
TM2937	<i>h⁻ leu1-32 ura4-D18 ade6-M216 his3-D1 rad3::ura4</i>	Laboratory stock
NR1593	<i>h⁻ leu1-32 ura4-D18 his3-237 rad1::ura4</i>	Laboratory stock
KT2785	<i>h⁻ leu1-32 ura4-D18 mrc1::ura4</i>	Laboratory stock
AS364	<i>h⁻ leu1-32 ddb1::hphMX6</i>	This study
AS355	<i>h⁻ leu1-32 ssb3::hphMX6</i>	This study
AS329	<i>h⁻ leu1-32 dcd1::kanMX6 ssb3::hphMX6</i>	This study
AS646	<i>h⁻ leu1-32 swi1::kanMX6</i>	This study
AS738	<i>h⁻ leu1-32 dcd1::kanMX6 swi1::kanMX6</i>	This study
SP896	<i>h⁻ leu1-32 ura4-D18 nse5::ura4⁺</i>	M. Boddy
AS743	<i>h⁻ leu1-32 ura4-D18 dcd1::kanMX6 nse5::ura4⁺</i>	This study
AS637	<i>h⁺ leu1-32 mus81::natMX6</i>	This study
AS860	<i>h⁺ leu1-32 dcd1::kanMX6 mus81::natMX6</i>	This study
PS2386	<i>h⁻ leu1-32 ura4-D18 rhp51::ura4⁺</i>	Laboratory stock
AS661	<i>h⁺ leu1-32 ura4-D18 dcd1::kanMX6 rhp51::ura4</i>	This study
OL4590	<i>h⁻ leu1-32 ura4-D18 ctp1::hphMx6</i>	Laboratory stock
AS664	<i>h⁺ leu1-32 ura4-D18 dcd1::kanMX6 ctp1::HphMX6</i>	This study
LLD3427	<i>h⁻ ura4-D18 leu1-32 chk1-9myc-2HA6His::ura4</i>	Laboratory stock
AS546	<i>h⁻ ura4-D18 leu1-32 dcd1::kanMX6 chk1-9myc-2HA6His::ura4</i>	This study
NB2118	<i>h⁻ ura4-D18 leu1-32 cds1-2HA6His::ura4</i>	Laboratory stock
AS536	<i>h⁻ ura4-D18 leu1-32 dcd1::kanMX6 cds1-2HA6His::ura4</i>	This study
EM4889	<i>h⁺ leu1-32 ura4-D18 rad11-GFP::hphMX6</i>	Laboratory stock
AS673	<i>h⁺ leu1-32 ura4-D18 rad11-GFP::hphMX6 dcd1::kanMX6</i>	This study
YYY4215	<i>h⁺ leu1-32 rad22-YFP::kanMX6</i>	Laboratory stock
AS701	<i>h⁺ leu1-32 rad22-YFP::kanMX6 dcd1::kanMX6</i>	This study
TMN3510	<i>h⁺ leu1-32::[hENT1 leu1⁺] ura4-D18 ade6-M210 his7-366::[hsv-tk his7⁺]</i>	S. Forsburg
AS873	<i>h⁺ leu1-32 ura4-D18 ade6-M210 his7-366::[hsv-tk his7⁺]</i>	This study
AS915	<i>h⁺ leu1-32 ura4-D18 ade6-M210 his7-366::[hsv-tk his7⁺] dcd1::kanMX6</i>	This study

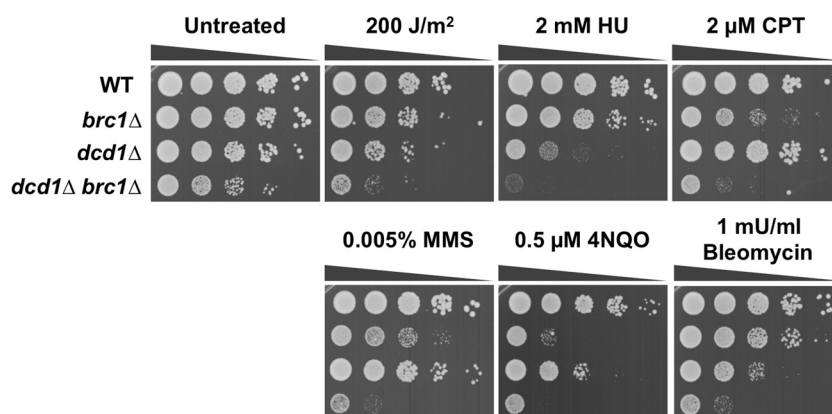


FIG 2 Genetic interaction between Dcd1 and Brc1. Tenfold serial dilutions of cells were exposed to the indicated DNA-damaging agents, and plates were incubated at 30°C for 3 to 4 days.

trifluoroethane; Aldrich, Sigma-Aldrich Sweden AB, Stockholm, Sweden] and 2.8 ml of >99% pure trioctylamine [Fluka, Sigma-Aldrich Sweden AB, Stockholm, Sweden]). The samples were vortexed and centrifuged for 1 min at 20,000 × *g*. The aqueous phase was collected and added to 700 µl of ice-cold Freon-trioctylamine mixture. Volumes of 475 and 47.5 µl of the aqueous phase were collected. The 475-µl aliquots of the aqueous phase were pH adjusted with 1 M NH₄HCO₃ (pH 8.9), loaded on boronate columns (Affi-Gel 601; Bio-Rad), and eluted with 50 mM NH₄HCO₃, pH 8.9, 15 mM MgCl₂ to separate dNTPs and NTPs. The eluates with purified dNTPs were adjusted to pH 3.4 with 6 M HCl, separated on a Partisphere SAX-5 high-performance liquid chromatography (HPLC) column (4.6 by 12.5 cm; PolyLC Inc., Columbia, MD), and quantified using a UV-2075 Plus detector (Jasco, Mölndal, Sweden). Nucleotides were isocratically eluted using 0.36 M ammonium phosphate buffer (pH 3.4; containing 2.5% [vol/vol] acetonitrile). The 47.5-µl aliquots of the aqueous phase were adjusted to pH 3.4 and used to quantify NTPs by HPLC in the same way.

Nitrogen starvation and release. Cells were grown to late-log growth phase in Edinburg minimal medium (EMM), harvested, and washed twice in EMM lacking nitrogen (EMM-N). Starvation and G₁ arrest were achieved by resuspending cells in EMM-N to a volume equal to that of the original culture and grown overnight at 25°C. Nitrogen was restored to half of the culture by transfer to a fresh flask and refeeding with an equal volume of EMM plus appropriate supplements and sampled every hour.

Flow cytometry. Cells were fixed in 70% ethanol and then treated with 0.1 mg/ml RNase A in 10 mM EDTA, pH 8.0, for at least 2 h at 37°C to eliminate RNA. Cells were stained with 1 µM SYTOX Green, sonicated, and analyzed using a FACSCalibur (Becton, Dickinson). Data analysis was carried out with Cell Quest software.

Immunoblots. For Cds1 and Chk1 shifts, whole-cell extracts were prepared from exponentially growing cells in standard NP-40 lysis buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 2.5 mM EDTA, 0.002% NP-40, 50 mM NaF, protease inhibitor tablet [Complete Mini; Roche]). Protein amounting to ~100 mg was resolved by SDS-PAGE using 10% gels with an acrylamide/bisacrylamide ratio of 99:1. Proteins were transferred to nitrocellulose membranes, blocked with 5% milk in Tris-buffered saline with 0.05% Tween, and probed with antihemagglutinin (12C5) antibody (Roche).

Microscopy. Cells were photographed using a Nikon Eclipse E800 microscope equipped with a Photometrics Quantix charge-coupled device (CCD) camera and IPLab Spectrum software. All fusion proteins were expressed at their own genomic locus. Rad52(Rad22)-yellow fluorescent protein (YFP)- and Rad11-green fluorescent protein (GFP)-expressing strains were grown in EMM until mid-log phase for focus quantification assays. Quantification was performed by scoring 500 or more nuclei from three independent experiments.

Mutagenesis assay. A mutation frequency assay was carried out as described previously (26). Yeast strains used in this assay contain the wild-type *ura4*⁺ gene at their endogenous locus. The strains were first grown on EMM plates without uracil to select for cells harboring wild-type *ura4*⁺. For analysis of spontaneous mutations, the *ura4*⁺ cells were plated on minimal medium containing uracil to allow accumulation of mutations. Colonies (≥11) were grown to late log phase in EMM containing 0.1 mg/ml uracil. Each culture (1 ml) was plated onto EMM agar plates containing 1 mg/ml 5-fluoro-orotic acid (FOA), 0.1 mg/ml uracil. FOA-resistant colonies (FOA^r) were counted after 7 to 10 days of growth. Total cells were determined by plating 100 µl of a 10⁻⁵ dilution onto EMM plates with 0.1 mg/ml uracil. Each assay was repeated at least 3 times. Mutation rates were calculated by fluctuation analysis using the Lea-Coulson method of the median by using the FALCOR program (20).

RESULTS

Brc1 is crucial in the absence of dCMP deaminase. Brc1 is required for survival of DNA-damaging agents that disrupt DNA replication (57). To gain new insights into Brc1, we carried out a genetic interaction screen known as epistatic mini-array profiles (E-MAP) (52) in which *brc1Δ* was combined with deletions of ~3,000 nonessential genes (28). The complete results of the screen will be described elsewhere; here, we focus on the negative genetic interaction between Brc1 and SPBC2G2.13c, which encodes a predicted dCMP deaminase that converts dCMP into dUMP, leading to the production of dTTP (Fig. 1). As SPBC2G2.13c is the ortholog of *S. cerevisiae* *DCD1*, we adopted the name *dcd1*⁺, but it should be noted that *dcd1*⁺ is also the obsolete name for *pim1*⁺, which encodes a Ran GDP/GTP exchange factor (63). We confirmed the genetic interaction between *dcd1Δ* and *brc1Δ* by creating and testing a new *dcd1Δ* null allele, which caused a modest growth defect that was substantially enhanced when combined with *brc1Δ* (Fig. 2, untreated). The *dcd1Δ* cells were mildly sensitive to 4-nitroquinoline N-oxide (4-NQO), which is consistent with a recent screen of the haploid deletion collection (11). The *dcd1Δ* cells were less sensitive to 4-NQO than *brc1Δ* cells, but they were noticeably more sensitive to hydroxyurea (HU), which inhibits DNA replication by inactivating RNR, and bleomycin, which causes double-strand breaks (DSBs). Unlike *brc1Δ* cells, the *dcd1Δ* mutant was largely resistant to the effects of the topoisomerase I inhibitor camptothecin (CPT) and the DNA alkylating agent methyl methanesulfonate (MMS), which cause replication fork stalling and collapse. Both *dcd1Δ* and *brc1Δ* mutants were

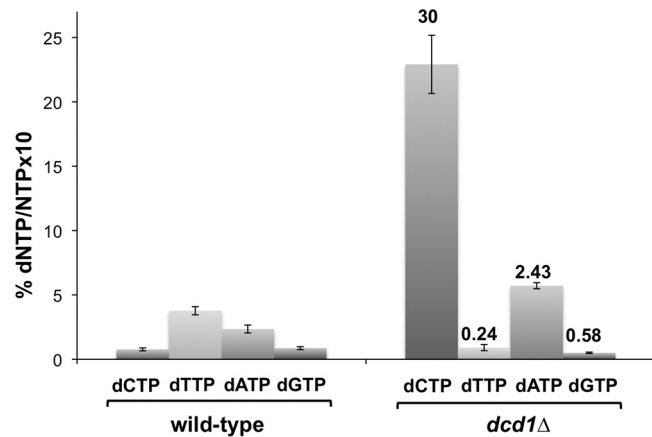


FIG 3 dCMP deaminase deficiency imbalances dNTPs pools. dNTP/NTP ratios are represented as means \pm SD. Numbers above the bars indicate fold increase or fold decrease relative to the wild type.

modestly sensitive to UV. Double mutant *dcd1Δ brc1Δ* cells appeared to be highly sensitive to many types of DNA-damaging agents. The negative genetic interactions between *dcd1Δ* and *brc1Δ* mutations imply that Dcd1 and Brc1 independently act in genome maintenance pathways that are partially complementary.

dCMP deaminase is required to maintain balanced pools of dNTPs. We used high-performance liquid chromatography to measure the cellular levels of dNTPs in log-phase cultures of wild-type and *dcd1Δ* cells (32). In the *dcd1Δ* mutant the dTTP concentration decreased 4-fold while that of dCTP increased 30-fold (Fig. 3). These data are consistent with dCMP deaminase diverting dNTP synthesis from dCTP to dTTP (Fig. 1) and with evidence that thymidylate depletion increases dCTP pools due to the lack of the feedback inhibition by dTTP on RNR (33). Smaller changes in dATP (2.5-fold increase) and dGTP (\sim 2-fold decrease) were also detected in the *dcd1Δ* mutant (Fig. 3).

Different DNA replication effects caused by dCMP deaminase deficiency versus reduction of RNR. We used flow cytometry to measure DNA content in log-phase *dcd1Δ* cultures (54). Whereas the haploid wild type had a single \sim 2C DNA peak because G_1 is very short and completion of S phase coincides with separation of daughter cells, the *dcd1Δ* mutant displayed a large enrichment of cells with a $<$ 2C DNA content, with a prominent peak indicating a cell cycle delay in G_1 or early S phase (Fig. 4). To investigate this defect further, we monitored DNA content in cells synchronously released from a G_1 -phase arrest imposed by nitrogen starvation (see Materials and Methods). Upon nitrogen re-feeding, progression into S phase in the *dcd1Δ* cells was delayed 1 additional hour relative to the wild type. This analysis also revealed that *dcd1Δ* cells were unable to efficiently arrest in G_1 phase when nitrogen starved, although very few cells appeared to arrest in S phase (Fig. 4). This *dcd1Δ* mutant phenotype may arise from an inability to complete S phase in a timely manner before nitrogen starvation halts growth, perhaps coupled with DNA damage checkpoint activation.

We performed a similar experiment with a *ddb1Δ* mutant, which is unable to degrade the RNR inhibitor protein Spd1 and has a more balanced \sim 3-fold decrease in all dNTP pools (23). Compared to *dcd1Δ*, this mutant displayed a less pronounced early S phase peak, but it had many more cells with an intermedi-

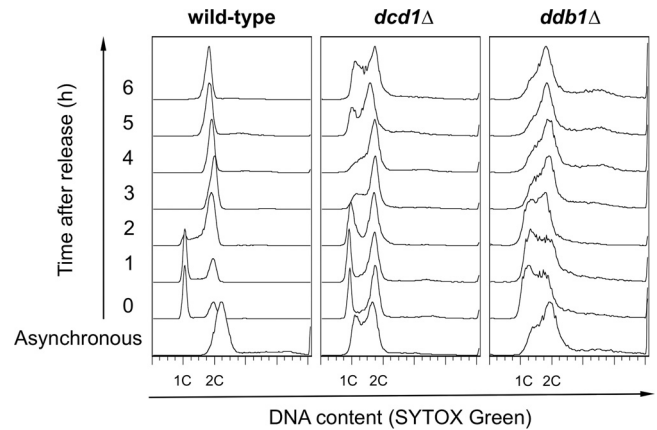


FIG 4 *dcd1⁺* deletion slows progression through S phase. Flow cytometer analysis of wild-type, *dcd1Δ*, and *ddb1Δ* strains. Cells were arrested in G_1 phase by nitrogen starvation for 16 h at 25°C, released from the block at 30°C in EMM containing nitrogen, and sampled every hour.

ate DNA content when starved of nitrogen. These effects are typical of cells treated with HU. The recovery kinetics from nitrogen starvation appeared to be similar in the two mutants. These data indicated that dCMP deficiency and failure to relieve inhibition of RNR have quite different effects on DNA replication.

Suppression of dCMP deaminase deficiency by expression of Herpes simplex virus thymidine kinase. Yeast cells are unable to salvage thymidine as they lack a thymidine kinase gene, therefore they completely rely on the *de novo* pathway to generate dTTP (Fig. 1) (19). A question raised by our studies is whether the phenotypes of *dcd1Δ* cells can be rescued by providing an alternative pathway for producing dTTP. We addressed this question by expressing the Herpes simplex virus thymidine kinase gene (*hsv-tk*), allowing conversion of thymidine to dTMP, which then enters the pathway of dTTP conversion (21, 58). Expression of *hsv-tk* in *dcd1Δ* cells suppressed their growth defect and restored resistance to UV, HU, 4NQO, and bleomycin (Fig. 5A). It also restored the flow cytometry profile of DNA content to a nearly normal pattern, with just a small number of cells in S phase (Fig. 5B).

Replication stress and DNA damage checkpoints are crucial in the absence of dCMP deaminase. Replication fork stalling activates the master checkpoint kinase Rad3 (ATR), which in turn activates the downstream effector kinase Cds1, which is required for recovery from replication fork arrest (51, 68, 69). We crossed *dcd1Δ* cells to *rad3Δ* and *cds1Δ* cells to assess the importance of the replication checkpoint. Tetrad analysis revealed that *dcd1Δ* was synthetically lethal with *rad3Δ* and *cds1Δ*. We also found that *dcd1Δ* was synthetically lethal with *mrc1Δ* (Table 2). Mrc1 is a checkpoint mediator protein required for activation of Cds1 by Rad3 (1, 62). To further probe the genetic interactions between Dcd1 and Cds1, we crossed *dcd1Δ* cells with the hypomorphic *cds1-T8A* mutant, which partially decreases Cds1 kinase activity and causes mild sensitivity to HU and other genotoxins that impair DNA replication (61). We observed an enhanced growth defect in the *dcd1Δ cds1-T8A* double mutant, which was further accentuated in cells treated with UV, HU, CPT, or MMS (Fig. 6C).

These data established that the Rad3-Mrc1-Cds1 signaling pathway is essential in *dcd1Δ* cells, suggesting that replication stalling in these cells activates the replication stress checkpoint. To

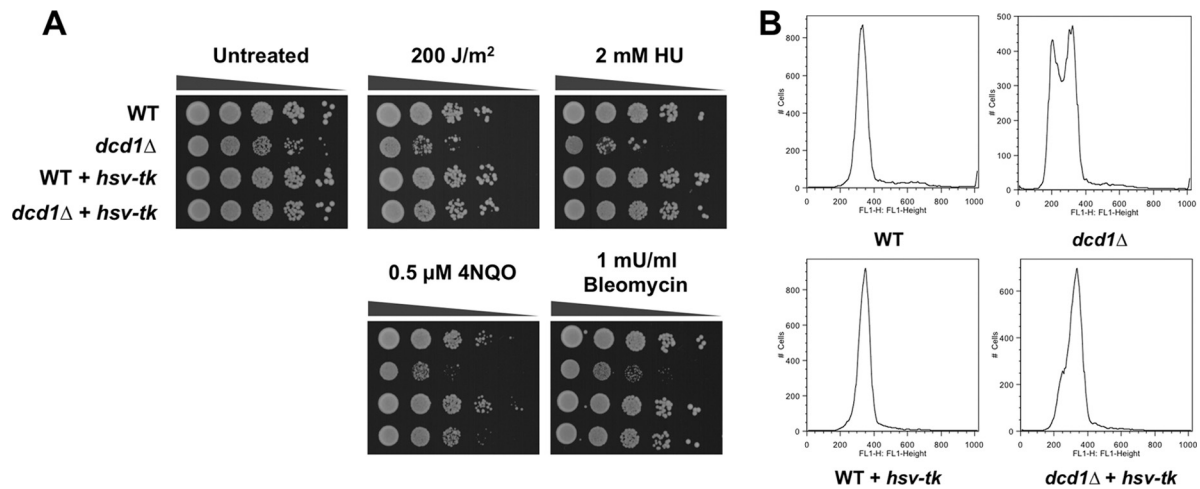


FIG 5 Suppression of *dcd1Δ* sensitivity phenotypes by expressing a herpes simplex virus thymidine kinase gene (*hsv-tk*). (A) Tenfold serial dilutions of cells were exposed to the indicated DNA-damaging agents, and plates were incubated at 30°C for 3 to 4 days. (B) Flow cytometer analysis of asynchronous, exponentially growing cultures of wild-type and *dcd1Δ* strains with or without expression of the *hsv-tk* gene.

address this question, we compared Cds1 phosphorylation in wild-type and *dcd1Δ* cells. Treatment of wild-type cells with HU triggers replication arrest and Cds1 activation as assessed by the appearance of a retarded electrophoretic mobility form of Cds1 (Fig. 6A). In *dcd1Δ* cells, phospho-Cds1 was detected even in the absence of HU treatment (Fig. 6A). These data indicated that Cds1 is activated in each cell cycle in the *dcd1Δ* mutant, which explains why Cds1 is essential in these cells.

Replication fork collapse creates one-ended DSBs that trigger Rad3 to activate Chk1, which is required to delay the onset of mitosis when DNA remains damaged after the completion of S phase. A strong negative genetic interaction was observed in *dcd1Δ chk1Δ* cells (Fig. 6D, untreated), which was enhanced in the presence of UV, HU, CPT, and MMS (Fig. 6D). These data suggested that increased spontaneous DNA damage in *dcd1Δ* cells activates Chk1. Indeed, immunoblot assays that detect phospho-Chk1 confirmed that Chk1 is activated even in the absence of genotoxin treatment in *dcd1Δ* cells (Fig. 6B). Taken together,

these data strongly indicate that abnormal dNTP pools in *dcd1Δ* cells lead to replication fork stalling and collapse.

Increased RPA and Rad52 foci in *dcd1Δ* cells. The Chk1 activation in *dcd1Δ* cells, and the strong negative genetic interaction between *dcd1Δ* and *chk1Δ*, suggested that unbalanced dNTP pools in *dcd1Δ* cells lead to increased spontaneous DNA damage. To test this proposition, we monitored Rad11-GFP foci in *dcd1Δ* cells. Rad11 is the *S. pombe* ortholog of RPA1, which is a subunit of replication protein A (RPA), which is the major single-strand DNA (ssDNA)-binding protein in eukaryotic cells (48, 66). We observed a large increase in cells with Rad11-GFP foci in the *dcd1Δ* strain (~35%) compared to the wild type (~9%), indicating increased replication fork stalling and/or spontaneous DNA damage (Fig. 7A). We also monitored Rad52-YFP foci in *dcd1Δ* cells. Rad52 (also called Rad22) is essential for DSB repair by homologous recombination (HR). Many mutants with genome maintenance defects have increased numbers of Rad52 foci (13, 41, 46). About 9% of wild-type cells had spontaneous Rad52-YFP foci,

TABLE 2 Summary of genetic interactions involving *dcd1Δ*^a

Allele	Function	Treatment				
		Untreated	UV	HU	CPT	MMS
<i>brc1Δ</i>	BRCT domain protein	Yes	YES	YES	YES	YES
<i>rad3Δ</i>	ATR checkpoint kinase	SL				
<i>chk1Δ</i>	DNA damage checkpoint kinase	YES	YES	YES	YES	YES
<i>cds1Δ</i>	DNA replication checkpoint kinase	SL				
<i>cds1.T8A</i>	DNA replication checkpoint kinase mutant	Yes	YES	YES	YES	YES
<i>mrc1Δ</i>	Mediator of replication checkpoint 1	SL				
<i>rad1Δ</i>	Checkpoint clamp complex protein	SL				
<i>ddb1Δ</i>	Damaged DNA binding protein	SL				
<i>ssb3Δ</i>	DNA replication factor A subunit	YES	YES	YES	YES	YES
<i>swi1Δ</i>	Replication fork protection complex subunit	YES	YES	YES	YES	YES
<i>nse5Δ</i>	Smc5-6 complex non-SMC subunit	YES	YES	YES	YES	YES
<i>mus81Δ</i>	Holliday junction resolvase subunit	YES	YES	YES	YES	YES
<i>rhp51Δ</i>	RecA family recombinase	YES	YES	YES	YES	YES
<i>ctp1Δ</i>	CtIP-related endonuclease	Yes	YES	YES	YES	YES

^a Double mutants were assessed for growth in the absence or presence of specified genotoxins. YES, strong negative interaction; Yes, negative interaction; SL, synthetically lethal.

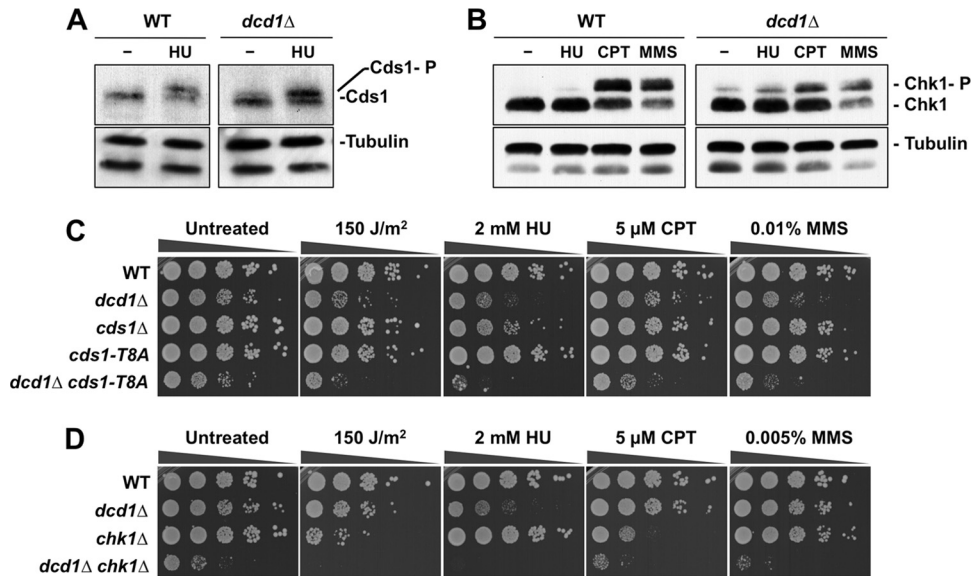


FIG 6 Replication and DNA damage checkpoint responses in *dcd1Δ* cells. (A) After HU treatment, Cds1 is phosphorylated in control and *dcd1Δ* cells, as indicated by the appearance of a slow-mobility species. Cds1 undergoes activating phosphorylation in *dcd1Δ* cells in the absence of damage. (B) After CPT and MMS treatment, Chk1 is phosphorylated in control cells, as indicated by the appearance of a slow-mobility species. Chk1 undergoes activating phosphorylation in untreated *dcd1Δ* cells. (C) Phenotype of *dcd1Δ* cells in combination with the *cds1-T8A* mutant. (D) Phenotype of *dcd1Δ* cells in combination with *chk1Δ*. Tenfold serial dilutions of cells were exposed to the indicated DNA-damaging agents, and plates were incubated at 30°C for 3 to 4 days.

whereas this percentage increased 2.3-fold (~21%) in *dcd1Δ* cells (Fig. 7B). These findings suggested that *dcd1Δ* cells suffer increased rates of spontaneous DNA damage.

Pathways required for recovery from replication fork collapse are crucial in the absence of Dcd1. These data suggested that *dcd1Δ* cells suffer increased rates of replication fork collapse, leading to formation of single-strand DNA and DNA repair foci and activation of the replication stress and DNA damage checkpoints. To further explore these effects, we performed a genetic epistasis analysis of *dcd1Δ* cells with genes involved in different pathways of DNA replication and repair. Double mutant strains were assessed for growth in dilution series on rich growth medium in the absence or presence of different genotoxins (Fig. 8). The data are summarized in Table 2. Deletion of the *dcd1⁺* gene is lethal in the absence of Rad1, which is a component of the 9-1-1

checkpoint clamp involved in the replication stress and DNA damage checkpoints (49). There was also a synthetic lethal interaction between *dcd1Δ* and *ddb1Δ*. As noted above, Ddb1 is required for the degradation of Spd1, which is an inhibitor of RNR (23). We also detected strong negative interactions with mutations that eliminate Ssb3, which is a small nonessential subunit of RPA (8); Swi1, a component with Swi3 of the replication fork protection complex (FPC), which stabilizes replication forks in a configuration that is recognized by replication checkpoint sensors (46); Nse5, a nonessential subunit of the Smc5-6 complex, which is crucial for recovery from replication-associated DNA damage (7, 50); Mus81, which associates with Emel to form a heterodimeric endonuclease that resolves Holliday junctions and is required for recovery from collapsed replication forks and resolution of crossovers in meiosis (4, 12, 18); and Rhp51, the Rad51

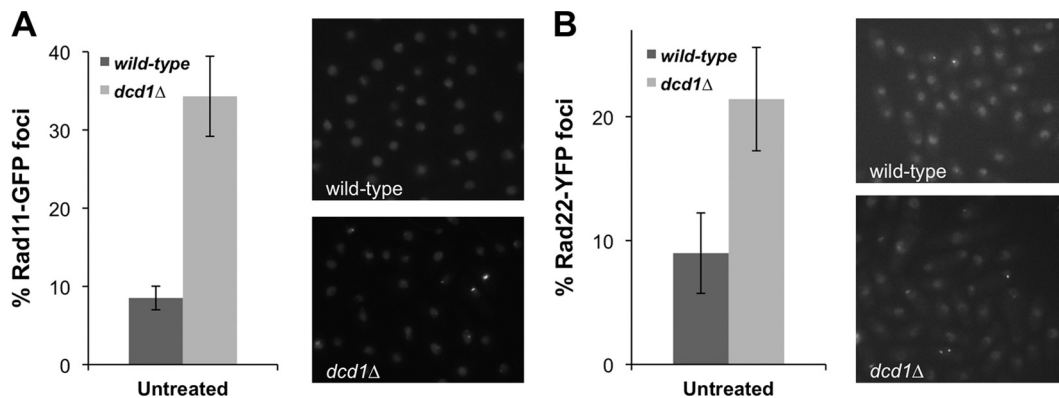


FIG 7 Rad11^{Rpa1} and Rad52 (Rad22) foci are increased in *dcd1Δ* cells. Rad11-GFP (A) and Rad22-YFP (B) form nuclear foci that increased in number in the absence of Dcd1. Cells expressing endogenous Rad11-GFP or Rad22-YFP in a wild-type and *dcd1Δ* background were cultured in minimal medium at 25°C until mid-log phase. Foci were scored in three independent experiments, and the mean values are represented. Error bars correspond to standard deviations of the means.

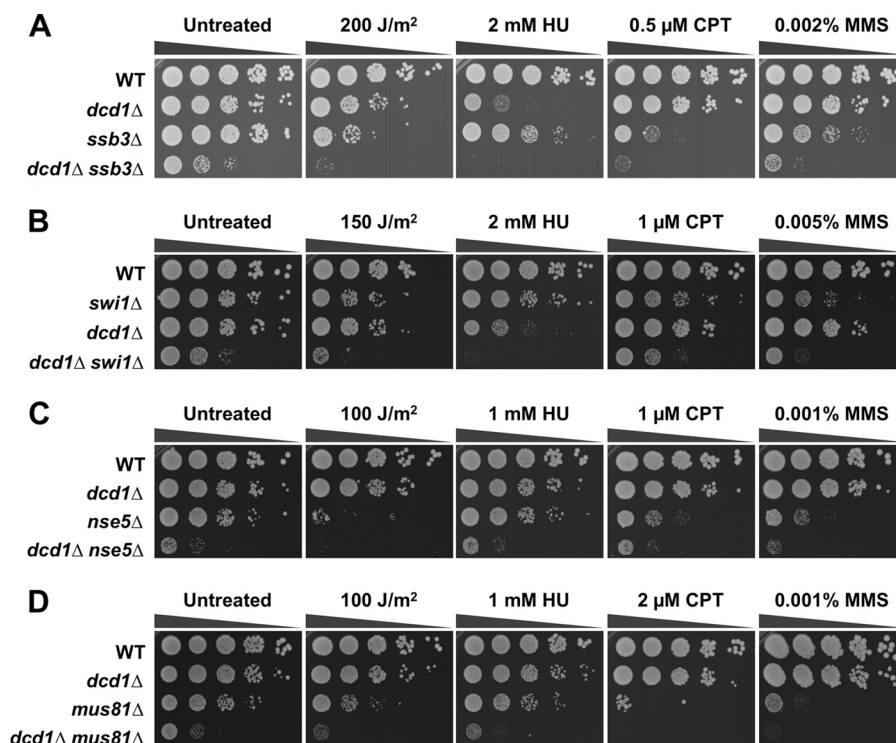


FIG 8 Genetic interactions involving Dcd1 and components required for recovery from replication fork collapse. Phenotypes of *dcd1Δ* in combination with *ssb3Δ* (A), *swi1Δ* (B), *nse5Δ* (C), and *mus81Δ* (D). Tenfold serial dilutions of cells were exposed to the indicated DNA-damaging agents, and plates were incubated at 30°C for 3 to 4 days.

ortholog required for most types of HR repair (44). We also detected a weaker negative genetic interaction with Ctp1, which is an HR repair factor required for the initiation of resection of DSBs prior to repair (35, 37).

All these data suggest that dCMP deaminase deficiency leads to replication fork stalling and collapse, necessitating requirements for genome maintenance pathways that stabilize replication forks and carry out the repair of collapsed replication forks.

Brc1 protects genome integrity in *dcd1Δ* cells. The phenotypes of *dcd1Δ* cells strongly suggest that dCMP deaminase is required for efficient DNA replication and repair, with the consequences of a dCMP deaminase defect being increased genetic instability. To directly test whether deletion of *dcd1*⁺ increases the spontaneous mutation rate, we used a mutation rate assay that detects mutations inactivating the *ura4*⁺ gene which confer resistance to FOA as described previously (26). Surprisingly, despite the acute dNTP pool imbalance in *dcd1Δ* cells, the mutation rate was unaffected (Table 3).

From these data, we hypothesized that other genome protection factors are important for reducing the spontaneous mutation rate in *dcd1Δ* cells. As a first step to investigate this possibility, we examined the role of Brc1. Whereas *brc1Δ*, like *dcd1Δ*, did not increase the spontaneous mutation rate, the *brc1Δ dcd1Δ* double mutant displayed an ~4-fold increase in spontaneous FOA-resistant colonies (Table 3). From these results, we conclude that Brc1 is required to maintain genome integrity in the cells lacking dCMP deaminase.

DISCUSSION

dCMP deaminase converts dCMP to dUMP, which is the nucleotide substrate for thymidylate synthase that is required for dTTP

synthesis. Its activity is allosterically regulated by the ratio of dCTP to dTTP, with dCTP as an activator and dTTP as an inhibitor (Fig. 1). With this central role in deoxyribonucleotide metabolism, dCMP deaminase should be crucial for efficient DNA replication and repair and for maintaining genome integrity. However, studies of *S. cerevisiae* suggest that eliminating dCMP deaminase has mild consequences, with the principal phenotype being a small increase in spontaneous mutagenesis (29). This mild phenotype contrasts with the profound consequences of decreasing RNR activity by chemical or genetic means. In this study, we have investigated dCMP deaminase in fission yeast, where we find that deleting the predicted dCMP deaminase gene increases dCTP ~30-fold and decreases dTTP ~4-fold. Expansion of the dCTP pool is likely due to the allosteric properties of RNR, which is regulated by dTTP but not dCTP, hence dCDP production was not diminished (33). Similar dNTP imbalances have been re-

TABLE 3 Effect of dCMP deaminase deficiency on spontaneous mutation rate^a

Strain	Mutation frequency ^b (per 10 ⁸ /generation)	CI	Ratio ^c
Wild type	3.9986	5.25–2.36	1.0
<i>rad2Δ</i>	41.1053	68.64–27.55	10.3
<i>dcd1Δ</i>	4.2736	6.11–3.22	1.1
<i>brc1Δ</i>	2.8495	3.78–1.74	0.7
<i>dcd1Δ brc1Δ</i>	15.1217	26.91–7.81	3.8

^a Mutation rates are the averages from three or more experiments. CI is the 95% confidence interval (10⁻⁸ per cell division).

^b Mutation rates were calculated by using the Lea-Coulson method of the median (20).

^c Ratio is relative to the wild type.

ported in other species (30, 39, 42, 64). Our key discovery is that dNTP imbalance in *dcd1Δ* cells delays progression in early S phase and creates critical requirements for key genome maintenance factors. These data establish that provision of balanced pools of dNTPs by dCMP deaminase is crucial for genome stability in fission yeast, in contrast to the situation in *S. cerevisiae*.

In many respects, the phenotypes of fission yeast *dcd1Δ* cells parallel the effects of decreased RNR activity. Notably, our studies revealed that *dcd1Δ* is synthetically lethal with mutations that delete Rad3, Cds1, and Mrc1, which are all critical for survival of HU treatment. Moreover, we found that *dcd1Δ* has a strong negative genetic interaction with *cds1-T8A*, which partially decreases Cds1 activity (61). The evidence indicating higher activation of Cds1 during the cell cycle in *dcd1Δ* cells supports these genetic data. These findings establish that the *dcd1Δ* mutant creates a requirement for a replication stress checkpoint. The dNTP imbalance in the *dcd1Δ* mutant, especially the low levels of dTTP, likely limits DNA synthesis and causes replication fork stalling, which leads to activation of the Rad3-Mrc1-Cds1 replication stress checkpoint. Also, this fork stalling may lead to replication fork collapse, triggering a DNA damage checkpoint, which explains why there is a strong negative genetic interaction between *dcd1Δ* and *chk1Δ*. Supporting this idea, *dcd1Δ* cells displayed higher activation of Chk1 in cycling cells and showed an elevated number of RPA and Rad52 foci, suggesting high rates of spontaneous DNA damage.

Indeed, *dcd1Δ* had negative genetic interactions with a large number of genes involved in genome protection, including Brc1, Ssb3, Mus81, Nse5, and Swi1. The negative genetic interaction with *brc1Δ* is noteworthy because Brc1 has an important role in promoting recovery from replication fork collapse (57, 65). The requirement for Mus81 in *dcd1Δ* cells is particularly informative, as besides its essential meiotic function, Mus81 is also required for recovery from a site-specific broken replication fork (4, 5, 53). It is thought that Mus81, in a heterodimeric complex with Eme1, cleaves Holliday junctions that are formed during reestablishment of a broken replication fork by homology-directed repair.

Although there are many parallels in the effects of dCMP deaminase and RNR deficiency in fission yeast, our data also suggest there are clear differences. In particular, we note that DNA content profiling of *dcd1Δ* cells reveals a prominent ~1C DNA peak that indicates a cell cycle delay in late G₁ or early S phase (Fig. 4). In contrast, cells with reduced RNR activity (e.g., *ddb1Δ* mutant or HU-treated wild-type cells) typically display a broader distribution of cells in S phase with DNA contents between 1C and 2C. An even more striking difference was observed in the DNA content profiles of nitrogen-starved *dcd1Δ* and *ddb1Δ* cells, in which the latter had many more cells arrested in S phase. These data suggest that dNTP pool imbalance in *dcd1Δ* cells inhibits entry into S phase, or, once having entered S phase and triggered a replication checkpoint response, they have more biochemical options for providing adequate supplies of dTTP to complete S phase. These options may operate more effectively in budding yeast, which could explain the mild phenotypes of *dcd1Δ* mutants in *S. cerevisiae*. Alternatively, the DNA polymerases in budding yeast may work more effectively with unbalanced pools of dNTPs.

The different phenotypes of *dcd1Δ* mutants in budding yeast and fission yeast suggest there are considerable variations in other species and cell types. In studies that predated molecular genetic characterizations, mouse S49 cells defective for dCMP deaminase had nearly normal doubling times with a somewhat prolonged

S phase (16), whereas V79 hamster fibroblasts lacking dCMP deaminase had almost twice the generation time of a control (3). We speculate that the viability of these cell lines has depended on the activation of checkpoint and DNA repair mechanisms, as we have observed with fission yeast.

Mouse S49 dCMP deaminase-deficient cells (16) exhibited a 10- to 20-fold increase in spontaneous mutation rates (64). Qualitatively similar results were observed in budding yeast, in which a *dcd1Δ* mutant had a 2.7-fold increase in spontaneous mutation rates (29), and in *Escherichia coli* infected with a dCMP deaminase deletion mutant of phage T4, in which mutagenesis increased 10- to 1,000-fold (56). The *E. coli* study observed a specific increase in AT→GC transition mutation. On the other hand, most of the mutations in the yeast study were GC→CG transversion mutations, an event that is not straightforwardly expected from the dNTP pool imbalance in these cells. These data indicate that mutagenesis induced by a DNA precursor pool imbalance involves factors other than concentration-dependent competition between nucleotides that are correctly and incorrectly base paired to a template base. In contrast, in a dCMP deaminase-defective hamster V79 cell line, the same dNTP imbalance had only a small effect on the spontaneous mutation rate (10). In our study, we found that the spontaneous mutation rate was unaffected in *S. pombe dcd1Δ* cells. However, protection against spontaneous mutations in *S. pombe dcd1Δ* cells depended on at least Brc1 and probably other genome maintenance factors. As seen for the viability of dCMP deaminase mutants, it is likely that mutation rates are kept in check by multiple DNA repair mechanisms, and these may play as important roles in mammalian cells as they do in fission yeast. In this regard, it will be interesting in future experiments to assess the spectrum of mutations in *brc1Δ dcd1Δ* cells.

Deoxycytidine analogs such as gemcitabine (2',2'-difluorodeoxycytidine) have strong antineoplastic activities that have led to their widespread use in clinical settings for treatment of solid tumors, including non-small-cell lung cancer, pancreatic cancer, bladder cancer, and breast cancer (24, 25, 34). The anticancer activity of gemcitabine is affected by metabolic inactivation through a pathway requiring dCMP deaminase. Thus, individual variation in dCMP deaminase activity likely influences the therapeutic response to treatment with gemcitabine, as well as to related nucleosides used in antiviral therapies. Moreover, dCMP deaminase may play an important role in tumors developing resistance to gemcitabine, which is a major problem in the clinic. The discovery of strong genetic interactions involving dCMP deaminase and genome maintenance factors in fission yeast, most of which are conserved in humans, may open up new opportunities for improving the therapeutic activities of deoxycytidine analogs.

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