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dUTPase activity is critical to maintain genetic stability in *Saccharomyces cerevisiae*

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ABSTRACT

We identified a viable allele (*dut1-1*) of the *DUT1* gene that encodes the dUTPase activity in *Saccharomyces cerevisiae*. The Dut1-1 protein possesses a single amino acid substitution (Gly82Ser) in a conserved motif nearby the active site and exhibits a greatly reduced dUTPase activity. The *dut1-1* single mutant exhibits growth delay and cell cycle abnormalities and shows a strong spontaneous mutator phenotype. All phenotypes of the *dut1-1* mutant are suppressed by the simultaneous inactivation of the uracil DNA *N*-glycosylase, Ung1. However, the *ung1 dut1-1* double mutant accumulates uracil in its genomic DNA. The viability of the *dut1-1* mutant is greatly impaired by the simultaneous inactivation of AP endonucleases. These data strongly suggest that the phenotypes of the *dut1-1* mutant result from the incorporation of dUMPs into DNA subsequently converted into AP sites. The analysis of the *dut1-1* strain mutation spectrum showed that cytosines are preferentially incorporated in front of AP sites in a Rev3-dependent manner during translesion synthesis. These results point to a critical role of the Dut1 protein in the maintenance of the genetic stability. Therefore, the normal cellular metabolism, and not only its byproducts, is an important source of endogenous DNA damage and genetic instability in eukaryotic cells.

INTRODUCTION

Abasic (AP) sites are thought to be one of the most frequent spontaneous lesions that occur in DNA, they are potentially lethal or mutagenic (1). In *Saccharomyces cerevisiae*, we proposed that three pathways cooperate to repair spontaneous AP sites and 3′-blocked single strand breaks (SSBs) resulting from the chemical or enzymatic (AP lyase) cleavage of AP sites in DNA: the two AP endonucleases/3′-phosphodiesterases (Apn1 and Apn2), the nucleotide excision repair (NER) and the 3′-flap endonuclease Rad1-Rad10 (1). In the absence of these three pathways, like in the *apn1 apn2 rad1* triple mutant, cells cannot support the burden of spontaneous AP sites and hence die (2–4). The origin of endogenous AP sites in DNA of living organisms is most probably diverse (1,5). Recent data show that an *apn1 apn2 rad1 ung1* quadruple mutant that is deficient in the unique Ung1 uracil DNA *N*-glycosylase in *S.cerevisiae* is viable, whereas an *apn1 apn2 rad1* mutant is not (4,6). Furthermore, the overexpression of the *DUT1* gene encoding the dUTPase (deoxyribouridine–triphosphate pyrophosphatase) restores the viability of an *apn1 apn2 rad1* triple mutant (6). These results point to the excision by Ung1 of uracil residues in U:A pairs, coming from the incorporation of dUMP in DNA by DNA polymerases during replication or repair, as a critical spontaneous source of AP sites in *S.cerevisiae* (6). The *DUT1* gene encodes the dUTPase (Dut1) that is required to convert dUTP into dUMP (deoxyribouridine-monophosphate), which is the unique precursor for *de novo* synthesis of dTTP (deoxyribothymidine-triphosphate) in yeast (Figure 1). In addition, Dut1 prevents the incorporation of dUMP into DNA, since DNA polymerases can efficiently use dUTP, even in the presence of dTTP, and incorporate it opposite adenine in DNA (Figure 1) (7–11). Therefore, alteration of the *DUT1* gene should challenge genetic stability in eukaryotes.

In *Escherichia coli*, the *dut* gene is essential (12), however mutant alleles such as *dut-1* have been isolated (13,14). The *dut-1* allele was found to have <1% of wild-type dUTPase activity (15). Although viable, the *dut-1* mutant exhibits a moderate spontaneous mutator phenotype, a high

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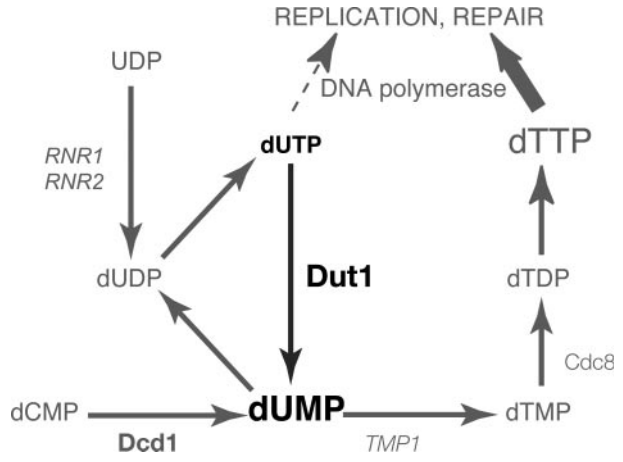

Figure 1. Biochemical pathways of dTTP biosynthesis in *S.cerevisiae*. Biosynthesis of dTTP is dependent upon dUMP synthesis. 60% of dUMP comes from the hydrolysis of dUTP by the dUTPase Dut1 and 40% comes from the deamination of dCMP by the dCMP deaminase Dcd1. DNA polymerases can use dUTPs or dTTPs during replication and repair and incorporate them opposite adenines yielding A:U pairs into DNA. The ratio dUTP/dTTP is maintained low by Dut1, so dTTP is incorporated in DNA much more frequently than dUTP (plain arrow versus dashed arrow, repectively). *RNR1,2*: ribonucleotide reductase genes, *TMP1*: Thymidilate synthetase gene, *CDC8*: Thymidilate kinase gene.

recombination frequency and synthetic lethality with mutations in the AP endonuclease gene *xth* or the homologous recombination gene *recA* (13,14,16,17). Furthermore, deletion of the uracil DNA *N*-glycosylase gene (*ung*) can suppress all the phenotypes associated with the *dut-1* allele and lead to the stable incorporation of a high level of uracil (~15–20% of total thymine residues) into DNA (15,18). These data strongly suggest that the synthetic lethality of the *xth dut-1* double mutant in *E.coli* is because of the excision by Ung1 of uracil incorporated by DNA polymerases into DNA.

In *S.cerevisiae,* like in *E.coli*, the *DUT1* gene is essential (19). Figure 1 shows that Dut1 is important for the biosynthesis of dTTP via the production of 60% of the dUMP pool. Dut1 is also essential to maintain the intracellular dUTP/dTTP ratio as low as possible to favor dTTP synthesis and to avoid incorporation of uracil into DNA during DNA replication (20). Indeed, the lethality of a null allele of *DUT1*, most probably relies on a high dUTP/dTTP ratio rather than to a defect in dTTP biosynthesis (19). It should be noted that dUTP pyrophosphatase is present in all living organisms with dTMP in their DNA and is also present in viruses (20–23). Furthermore, several studies indicate that the relative expression levels of both dUTPase and uracil DNA *N*-glycosylase can have great influence over the efficacy of thymidilate synthase-directed cancer chemotherapy (24,25).

Our recent data point to AP sites that result from the excision of uracil incorporated into DNA in the course of replication and repair as an important source of endogenous DNA damage in *S.cerevisiae* (6). In this model, the *DUT1* gene coding for the dUTPase, Dut1, plays a central role in the prevention of the formation of endogenous DNA damage. *DUT1* is an essential gene, so it is not possible to analyze its impact on genetic stability using a deletion mutant. Therefore, it was critical to generate a viable allele of *DUT1* with a compromised dUTPase activity. In this study, we present the isolation of such a mutant, called *dut1-1*. We showed that this mutant exhibits growth delay and cell cycle abnormalities. In addition, the *dut1-1* mutation is highly deleterious in AP sites repair deficient strains. Finally, the *dut1-1* mutant exhibits a robust spontaneous mutator phenotype that is Rev3 and Ung1-dependent. Taken together, these data show, for the first time, that the Dut1 protein plays an important role in the maintenance of the genetic stability in eukaryotic cells.

MATERIALS AND METHODS

Yeast culture and genetic procedures

Yeast strains were grown at 30°C in YP or YNB medium supplemented with appropriate amino acids and bases and 2% glucose (YPD or YNBD medium) or 2% galactose (YPGal or YNBGal medium). 5-FOA drug was added at 750 μg/mL in YNB complemented with all amino acids. All media including agar were from Difco. Pre-sporulation and sporulation procedures were performed as previously described (26). Micromanipulation and dissection of asci were performed with a Singer MSM system (27). Yeast strains were transformed using a lithium acetate method as previously described (28).

Yeast strains and plasmids

The *xth* gene of *E.coli* was amplified by PCR and cloned into p414GAL1 (29) yielding p414GAL1-*xth*. GAL1-*xth* was amplified from p414GAL1-*xth* and cloned into an *ADE3* plasmid, pCH1122 (30), yielding pADE3-GAL1-*xth*. The DNA library used during the screen is a genomic library *URA3* obtained from Dr F. Lacroute (Sau3AI genomic fragments of 5–10 kb cloned into Yep24 at BamHI site) (31,32). Primers used for PCR amplification, detailed cloning strategies and DNA sequencing analyses are available upon request. *S.cerevisiae* strains used in this study are isogenic to FF18733 and listed in Table 1. Deletion of *APN1*, *APN2* and *UNG1* were previously described (6). *ADE3* gene was deleted by transformation of the CS37 strain (*ade2*Δ) with the plasmid p368 (gifts from Dr F. Fabre), yielding BG187. *DUT1* was deleted in a diploid WT strain (BG213) using a PCR-mediated one step-replacement technique (33) yielding BG199, only the 5′ half of the gene was deleted not to interfere with the expression of the neighboring essential gene *SRB6*. The inactivation of *DUT1* was checked by sporulation of BG199, after dissection, only two spores were growing in each tetrad confirming the essential character of *DUT1* (19). BG199 was transformed with p424GAL1-*DUT1* [previously described (6)], and sporulated to obtain a single *dut1*Δ mutant carrying p424GAL1-*DUT1*, called BG218 / p424GAL1-*DUT1*. This result confirmed that the spore lethality obtained after sporulation and dissection of BG199 is specific of the deletion of *DUT1* in BG199. BG218/p424GAL1-*DUT1* was then crossed with BG135, the diploid was grown on YPD and a colony that lost the plasmid was selected yielding BG235. BG201 was obtained by crossing BG41 with BG187 and a colony that lost the *URA3* auxotrophy was selected on 5-FOA plate. BG201 was then transformed with p*ADE3*-GAL1-*xth* yielding BG201/p*ADE3*-GAL1-*xth* used to perform the screen.

Table 1. *S.cerevisiae* strains used in this study

Strains	Genotype	References
FF18733	*MAT**a** leu2-3,112 trp1-289 his7-2 ura3-52 lys1-1*	F. Fabre
FF18734	*MATα leu2-3,112 trp1-289 his7-2 ura3-52 lys1-1*	F. Fabre
FF181134	FF18733 with *rev3Δ::URA3*	Padula *et al.* (34)
CS37	FF18733 with *ade2Δ*	F. Fabre
BG3	FF18733 with *apn1Δ::URA3*, *apn2Δ::kanMX6*	Guillet and Boiteux (2)
BG40	FF18733 with *apn1Δ::URA3*, *apn2Δ::kanMX6, rad14Δ::LEU2*	Guillet and Boiteux (6)
BG41	FF18734 with *apn1Δ::URA3*, *apn2Δ::kanMX6*, *rad14Δ::LEU2*	This study
BG135	FF18733 with *ung1Δ::URA3*	Guillet and Boiteux (6)
BG187	FF18733 with *ade2Δ*, *ade3Δ*	This study
BG199	Diploid with *dut1Δ::URA3/DUT1*	This study
BG201	FF18733 with *ade2Δ*, *ade3Δ*, *apn1Δ::ura3*, *apn2Δ::kanMX6, rad14Δ::LEU2*	This study
BG201/p*ADE3-GAL1-xth*	FF18733 with *ade2Δ*, *ade3Δ*, *apn1Δ::ura3*, *apn2Δ::kanMX6, rad14Δ::LEU2*; with p*ADE3*-GAL1-*xth*	This study
BG213	Diploid wild type	This study
BG216/ p*ADE3-GAL1-xth*	FF18733 with *ade2Δ*, *ade3Δ, apn1Δ::ura3, apn2Δ::kanMX6, rad14Δ::LEU2*, *dut1-1*; with p*ADE3*-GAL1-*xth* from the screen	This study
BG216/p*414GAL1-xth*	FF18733 with *ade2Δ*, *ade3Δ, apn1Δ::ura3, apn2Δ::kanMX6, rad14Δ::LEU2*; *dut1-1*; with p414GAL1-*xth*	This study
BG217	FF18734 with *dut1-1*, *ade2Δ*	This study
BG217/p424GAL1	FF18734 with *dut1-1*; with p424GAL1	This study
BG217/p424GAL1-*DUT1*	FF18734 with *dut1-1*; with p424GAL1-*DUT1*	This study
BG218/ p424GAL1-*DUT1*	FF18734 with *dut1Δ::URA3/DUT1*; with p424GAL1-*DUT1*	This study
BG228	FF18733 with *apn1Δ::URA3*, *apn2Δ::kanMX6 dut1-1*	This study
BG234	FF18733 with *dut1-1*, *ung1Δ::URA3*	This study
BG235	Diploid with *dut1 Δ::URA3/DUT1*, *ung1Δ::URA3/UNG1*	This study
BG239	FF18733 with *dut1-1*, *rev3Δ::URA3*	This study

p*ADE3*-GAL1-*xth* was exchanged with p414GAL1-*xth* by plasmid shuffling on 5-FOA in BG216 to allow the transformation of BG216/p414GAL1-*xth* with a URA3 library (see below). BG135 and FF181134 were crossed with BG217 and after sporulation of the diploids, the strains BG234 and BG239 were selected, respectively. For all the mutants in this study containing the *dut1-1* mutation, the *DUT1* gene was amplified by PCR and sequenced as explained below. All strains used in this study are available from the authors.

Sequencing of the *DUT1* gene

The *DUT1* gene was amplified by colony PCR using the primers *DUT1* flk5′ (ATGACTGCTACTAGCGACAAAGTA) and *DUT1* flk 3′ (TTAGTTACCAGTGCTACCAAAGCC) and the oligonucleotide *DUT1* flk 5′ was used to sequence the gene using a Pharmacia kit®. The sequences were analyzed with an ALFexpress automated sequencer (Amersham Pharmacia Biotechsize).

Flow cytometry analysis and DAPI staining

Flow cytometry analysis and DAPI (4,6-diamidino-2-phenylindole) staining were done as previously described (6).

Spontaneous mutation frequencies and mutation spectra

Yeast strains were grown in 2 ml of YPD medium at 30°C. The *dut1-1* strains transformed with p424GAL1-*DUT1* (BG217/p424GAL1-*DUT1*) or p424GAL1 (BG217/p424GAL1) were grown in YNBGal medium supplemented with appropriate amino acids and bases at 30°C. Spontaneous mutation frequencies and mutation spectra were performed as previously described (34).

Preparation of cell-free extracts and assay for dUTPase activity

Cell-free extracts and dUTPase assay were performed as described (6). One unit of dUTPase activity releases 1 pmol of dUMP per minute at 37°C.

Measurement of uracil in genomic DNA

Yeast strains were grown at 30°C in YPD medium (200 ml) until $OD_{600} = 1.0$. Afterwards, cells were harvested, washed in water, pelleted and stored at −80°C. Genomic DNA was extracted as follows. Yeast cells were resuspended in 1 ml of zymolyase solution (3 mg/ml zymolyase, 1 M sorbitol, 0.1 M EDTA) and incubated for 1 h at 37°C. Following a 5 min centrifugation at 8000 r.p.m., the pellet was resuspended in 1 ml of TE buffer (20 mM Tris–HCl pH 8.0, 50 mM EDTA) and incubated for 45 min at 65°C in the presence of SDS and proteinase K (final concentration 0.3% and 0.2 mg/ml, respectively). Then, DNA was ethanol-precipitated, resuspended in TE buffer and submitted to RNase treatment. Finally, DNA was precipitated and resuspended in TE buffer and kept at 4°C. Aliquots (10 μg) of DNA were incubated for 45 min at 37°C in a reaction buffer (12 μl-final volume) that contained 0.6 μg of *E.coli* Ung1 protein (our laboratory stock). Prior to loading on a 0.6% alkaline agarose gel, samples were denatured for 20 min at room temperature by adding 10 μl of 100 mM NaOH, 4 mM Na_2EDTA and 10 μl denaturing gel loading buffer (50% glycerol, 1N NaOH, 0.2% bromocresol green). Electrophoresis was run overnight at 1.3 V/cm in the cold room at 4°C. Later the gel was neutralized, stained with ethidium bromide at 1 mg/l and washed with water. Finally, fluorescence was measured using a Typhoon-9400 apparatus (Amersham-Bioscience).

RESULTS

Isolation of the *dut1-1* allele of the *DUT1* gene encoding Dut1-G82S

To isolate mutations in genes that control the formation or repair of endogenous AP sites in *S.cerevisiae*, we performed a co-lethality screen using a red/white color colony assay as previously described (35). We searched for mutations that impair the viability of an *apn1 apn2 rad14* triple mutant (36). Although viable, an *apn1 apn2 rad14* mutant exhibits an extreme sensitivity to the lethal action of agents that generate AP sites such as methyl-methanesulfonate (MMS) [(37) and Figure 2A]. We expected that mutations in genes *(x)* that affect the formation or repair of endogenous AP sites will cause cell death in the *apn1 apn2 rad14 (x)* quadruple mutants. Since our recent data pointed out the incorporation of dUMP from dUTP pool in DNA as an important source of endogenous AP sites, this assay was suitable for the isolation of mutations in the *DUT1* gene. The co-lethality screen was performed as follows (Figure 2B). First, we expressed the *xth* gene encoding the major AP endonuclease of *E.coli* under the control of a GAL1 promoter on a centromeric yeast plasmid carrying the *ADE3* gene (p*ADE3*-GAL1-*xth*) in the BG 201 (*apn1 apn2 rad14 ade2 ade3*) mutant yielding BG 201 / p*ADE3*-GAL1-*xth*. Figure 2A shows that, in the presence of galactose, the expression of *xth* suppresses the hypersensitivity to MMS of the *apn1 apn2 rad14 ade2 ade3* mutant with respect to the killing effect of MMS. We expected that the AP endonuclease/3′-phosphodiesterase activity of Xth will suppress the lethality of quadruple mutants harboring a mutation in a gene that is synthetic lethal with *apn1*, *apn2* and *rad14*. Second, we exposed the BG 201/ p*ADE3*-GAL1-*xth* strain to ultraviolet-light at 260 nm (8 J m^{-2}) to generate mutants. Third, we screened for plasmid-dependent colonies by visualizing their color on complete medium with galactose (YPGal plates), we selected red non-sectored colonies (Figure 2B). Furthermore, since the *xth* gene was under the control of a GAL1 promoter the candidates should have a strong growth defect on complete medium with glucose (YPD plates) but not on YPGal plates (Figure 2A and 2B). Out of 22 000 clones screened, four mutants were retained after both the selections, red non-sectoring colonies and very poor growth on YPD. Among these four candidates, the growth defect of one of them, BG216 / p414GAL1-*xth* (obtained by plasmid shuffling, see Materials and Methods) was suppressed on glucose containing plates by plasmids carrying overlapping genomic inserts containing the *DUT1* gene. Furthermore, the genomic *DUT1* gene

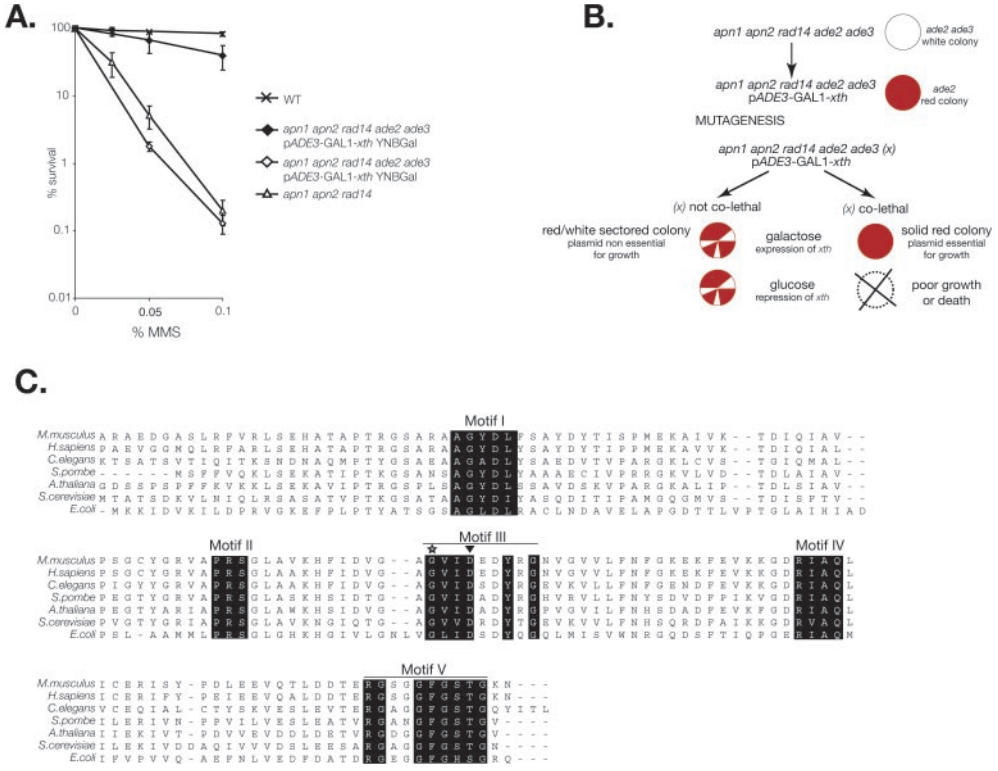

Figure 2. Isolation of the *dut1-1* allele. (**A**) The *apn1 apn2 rad14 ade2 ade3* / p*ADE3*-GAL1-*xth* strain (BG201 / p*ADE3*-GAL1-*xth*) used in the co-lethality screen and the control strains were grown to exponential phase in YNB medium containing glucose (YNBGlc) or galactose (YNBGal) and exposed to different concentration of MMS. Experimental points are the average of at least three experiments. The WT strain (FF18733) and the *apn1 apn2 rad14* mutant (BG40) are shown as controls. (**B**) A red/white color colony assay was performed using the strain BG201, *apn1 apn2 rad14 ade2 ade3* (white colony). First, BG201 was transformed with p*ADE3*-GAL1-*xth* (red colony). Second, BG201 / p*ADE3*-GAL1-*xth* was mutagenized using UV. Finally, co-lethal mutations were isolated by selection of strains that (i) were not able to lose the plasmid (solid red colonies) and (ii) were viable on complete medium containing galactose, when *xth* is expressed, but not on complete medium containing glucose, when *xth* is repressed (right side). In contrast, if the mutations are not co-lethal, the strains (i) can lose the plasmid carrying *ADE3* and present red/white sectored colonies and (ii) are viable on complete medium containing glucose (left side). (**C**) Sequence alignments of dUTPases in seven organisms. Conserved residues within the five (I–V) dUTPase motifs are in white on black background (38). The arrowhead points at the aspartic acid essential for the catalytic activity of the dUTPase and the star shows the G82S mutation of the *dut1-1* mutant.

in the selected mutant was sequenced and revealed a base substitution at position 244, a transition GC to AT, yielding an amino acid substitution at position 82 in the Dut1 protein, from a glycine to a serine (G82S). The *DUT1* gene was sequenced in the parental strain and in the three other mutants but no mutation was found compared with WT. Sequence alignment of Dut1 in different organisms revealed that the G82 residue mutated is evolutionary conserved from *E.coli* to human (Figure 2C) and is next to the aspartic acid residue essential for the dUTPase activity (38). These data point to an impaired dUTPase activity in cells harbouring the *dut1-1* allele.

The *dut1-1* allele is deleterious in WT strain and AP sites repair defective mutants

The *dut1-1* single mutant was obtained by backcrossing the *apn1 apn2 rad14 ade2 ade3 dut1-1* (BG216/p414GAL1-*xth*) mutant selected through the screen with an isogenic WT strain (FF18734). The *DUT1* gene was sequenced to identify the *dut1-1* mutation in spores that are *APN1* [checked by PCR as previously described (2)], *APN2* (G418 sensitive) and *RAD14* (leucine auxotrophy) and that do not carry p414GAL1-*xth* (uracil auxotrophy). This first *dut1-1* single mutant was then backcrossed twice with an isogenic WT strain to obtain the final *dut1-1* mutant used in this work called BG217. For each full tetrad obtained in *dut1-1*×WT crosses (total number of tetrads = 16), 2 spores presented a slight growth defect compared with a WT cross (data not shown). The *DUT1* gene was sequenced for the slow growing spores (three tetrads, six *dut1-1* putative mutants) and all of them possessed the *dut1-1* mutation. The growth defect after germination was then confirmed by measuring the division time in complete medium (Table 2). In exponential culture in rich medium, the *dut1-1* mutant also accumulates large budded cells with the nucleus at the bud neck, 32% for *dut1-1* compared with 12% for WT (Table 2). Moreover, the FACS analysis shows an accumulation of cells with 2N content and a shift on the right that can be explained by the presence of dead cells (Figure 3A). The reduced plating efficiency of a *dut1-1* mutant (44%) compared with the WT (80%) confirms the presence of dead cells in exponentially growing cultures. All these experiments were done at 30°C; we did not observe any cryosensitivity or thermosensitivity of the *dut1-1* mutant (data not shown). It should be noted that the *dut1-1* strain is not unusually sensitive to the lethal action of genotoxic agents like MMS, UVC radiation or gamma-radiation, compared with the WT strain (data not shown). These results strongly suggest that *DUT1* is not part of a repair or a checkpoint pathway but is probably involved in the accumulation of DNA damages that trigger a G2/M checkpoint.

To confirm the co-lethality of the *apn1 apn2 rad14 dut1-1* mutant obtained with the screen, the *dut1-1* mutant (BG217) was crossed with an *apn1 apn2 rad14* triple mutant (BG40), and the products of dissection have been identified and characterized (Figure 3B and Table 2). The tetrad analysis showed that the presence of the *dut1-1* mutation causes a severe growth defect in cells whose capacity to repair AP sites was impaired, namely the *apn1 apn2 rad14 dut1-1* (Figure 3B, spore 4A versus 3D), *apn1 apn2 dut1-1* or BG228 (Figure 3B, spore 3B versus 1C, 2B, 5D or 6A) and *apn1 rad14 dut1-1* (Figure 3B, spore 1D versus 2C or 5C) mutants, with the exception of the *apn2 rad14 dut1-1* mutants (Figure 3B, spor 6D versus 4B). It should be noted that the growth defect of the *apn1 apn2 rad14 dut1-1* mutant obtained with this cross was extreme and very similar to the one of the mutant selected in the course of our co-lethal screening (BG216/p*ADE3*-GAL1-*xth*) on glucose containing plate. This result strongly suggests that the *dut1-1* mutation is the cause of the observed phenotype. Taken together, division time, FACS analysis and microscopic examinations of the cells of these various mutants confirmed the deleterious impact of the *dut1-1* mutation in AP sites repair defective mutants (Table 2).

Table 2. Deleterious effect of the *dut1-1* mutation in mutant defective in AP sites repair

Strains	Division time (min)[a]	Percentage of large budded cells[b]	Percentage of large budded cells with nucleus at the bud neck[c]
WT	96 ± 7	35 ± 4	12
dut1-1	144 ± 18	55 ± 11	32
apn1 apn2	109 ± 5	43 ± 11	20
apn1 apn2 dut1-1	175 ± 32	70 ± 7	66
apn1 apn2 rad14	117 ± 7	53 ± 7	46
apn1 apn2 rad14 dut1-1[d]	316 ± 75	n.d.	n.d.
apn1 rad14	119 ± 11	38 ± 2	45
apn1 rad14 dut1-1	219 ± 23	67 ± 9	81
apn2 rad14	111 ± 6	36 ± 6	21
apn2 rad14 dut1-1	129 ± 5	43 ± 8	38

n.d.: not determined.
[a]The cells were grown in YPD at 30°C and the division time was calculated during the exponential phase of the growth. The times correspond to the average and standard deviation of at least two independent cultures.
[b]The percentage of large budded cells correspond to the percentage of cells with a bud at least two-third the size of the mother cell. The cells were counting in exponential culture grown at 30°C. The numbers indicated represent the average and standard deviation of three independent experiments, at least 200 cells were counted for each of them.
[c]The percentage of large budded cells with the nucleus at the bud neck was estimated by DAPI staining of cells grown in YPD at 30°C to exponential phase. The results indicated are the average and SD of at least 2 independent cultures. At least 100 cells were counted for each experiment.
[d]The cells are too sick to determine the percentage of large budded cells and the percentage of large budded cells with the nucleus at the bud neck.

The Dut1-G82S protein has an impaired dUTPase activity *in vitro* and the dut1-1 mutant incorporates high levels of uracil into genomic DNA *in vivo*

Our data suggest that the dUTPase activity of the Dut1-G82S protein must be altered compared with the WT. To test this hypothesis, we measured the dUTPase activity in cell-free extracts of exponentially growing WT and *dut1-1* strains (6). Table 3 shows that the dUTPase activity is greatly diminished in the *dut1-1* mutant, it represents <10% of the WT activity. Therefore, the glycine 82 is important for the dUTPase activity of Dut1 as previously suggested (Figure 2C and Table 3).

If the dUTPase activity is decreased in a the *dut1-1* strain, the ratio dUTP/dTTP is presumably increased and the DNA polymerases incorporate dUTP instead of dTTP opposite adenine yielding an increased number of U:A pairs in this mutant (Figure 1). To evaluate the amount of uracil

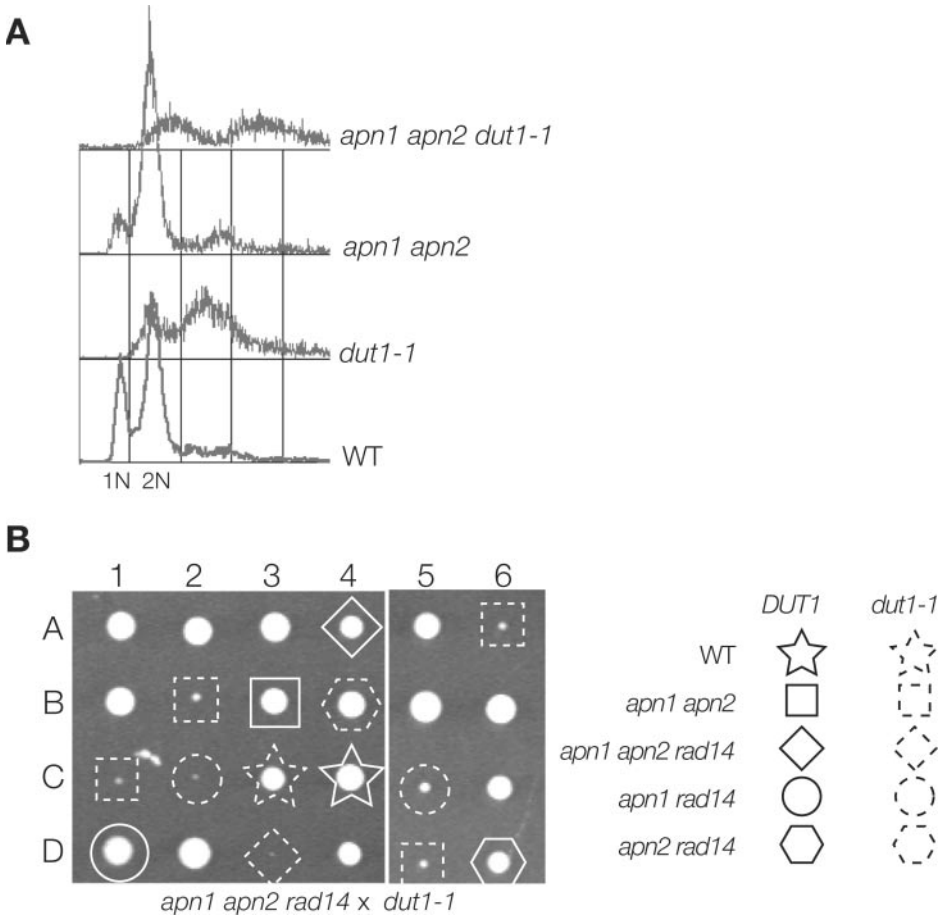

Figure 3. The deleterious effect of the *dut1-1* allele in strains defective in AP sites repair. (**A**) WT (FF18733), *dut1-1* (BG217), *apn1 apn2* (BG3) and *apn1 apn2 dut1-1* (BG228) strains were grown in YPD medium at 30°C to exponential phase, fixed and analyzed by FACS as described in material and methods. (**B**) The strain *dut1-1* (BG217) was crossed with a triple mutant *apn1 apn2 rad14* (BG40) and the diploid strain obtained was sporulated. The figure shows a selection of tetrads with spores containing the *dut1-1* mutation (dashed line) and the same mutants with the WT *DUT1* gene (same shape with plain line).

Table 3. Phenotypes of *dut1-1* mutant in combination with *ung1* deletion

Strains	Division time[a]	percentage of large budded cells[a]	percentage of large budded cells with nucleus at the bud neck[a]	Spontaneous mutagenesis CanR/10^7 cells[b]	dUTPase activity (U)
WT	93 ± 3	37 ± 3	28	10	2320
dut1-1	154 ± 18	60 ± 4	66	455	⩽100
dut1-1 + p414GAL1-DUT1	n.d.	n.d.	n.d.	11	n.d.
dut1-1 + p414GAL1	n.d.	n.d.	n.d.	196	n.d.
ung1	85 ± 3	38 ± 4	33	38	2380
ung1 dut1-1	123 ± 21	49 ± 4	46	53	170
dut1-1 rev3	175 ± 32	n.d.	n.d.	11	n.d.

n.d.: not determined.
[a]Measurements were performed as described in the legend of Table 2.
[b]The spontaneous mutation frequencies correspond to the median of at least 33 independent cultures.

incorporated in the genomic DNA of the *dut1-1* mutant, the U:A pairs must be protected from the action of the only uracil DNA *N*-glycosylase, Ung1 (39,40). Thus, we crossed the *dut1-1* mutant (BG217) with a strain deleted for the *UNG1* gene (BG135). We first compared the phenotypes of the *ung1 dut1-1* double mutant (BG234) with that of the *dut1-1* or *ung1* single mutants. Table 3 shows that the division time, the number of large budded cells and the number of large budded cells with the nucleus at the bud neck of the *dut1-1 ung1* mutant are decreased compared with the *dut1-1* mutant. Therefore, the *ung1* mutation alleviates the growth defects of the *dut1-1* strain. In addition, FACS analysis showed that there is no more accumulation of G2 cells in the *ung1 dut1-1,* which exhibits a profile identical to that of the WT strain (data not shown). As expected, the dUTPase activity in the *ung1 dut1-1* is low, like in a *dut1-1* single mutant (Table 3). These

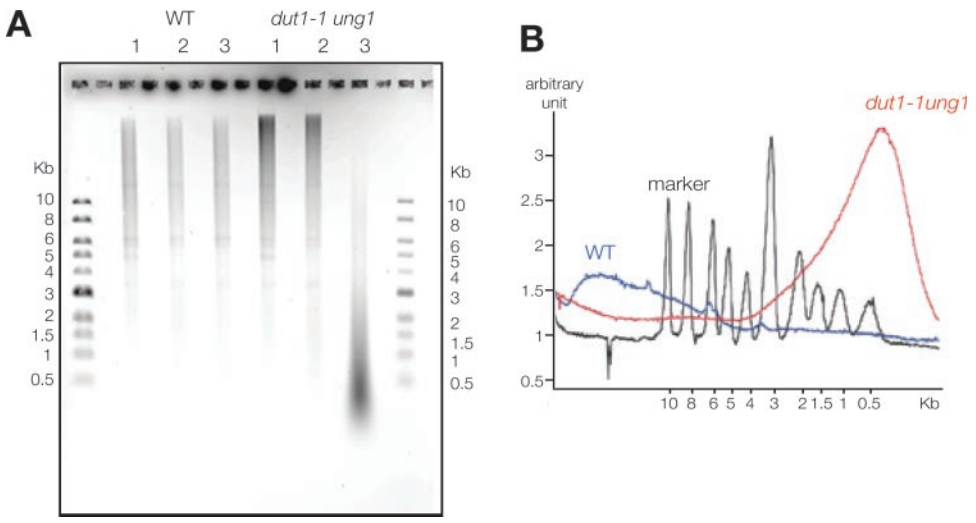

Figure 4. The *dut1-1 ung1* mutant accumulates uracil in genomic DNA. (**A**) Genomic DNA of exponential growing WT (FF18733) and *ung1 dut1-1* (BG234) cells was extracted, treated with uracil DNA *N*-glycosylase from *E.coli* (Ung) and under alkaline condition to break DNA at AP sites before loading on an agarose gel as described in Materials and Methods. Lane 1: untreated DNA. Lane 2: DNA incubated for 30 min at 37°C without Ung. Lane 3: DNA incubated for 30 min at 37°C in the presence of 0.6 µg of Ung protein from *E.coli*. (**B**) Estimation of the length of the DNA fragments obtained after treatment with Ung of the genomic DNA of a WT (FF18733) and *ung1 dut1-1* (BG234) strain. Lanes 3 (WT and *ung1 dut1-1*) as well as marker lane were scanned as described in Materials and Methods.

results show that the deletion of *UNG1* suppresses the phenotype of slow growth and G2/M arrest of the *dut1-1* mutant. Taken together, this data strongly suggests that the repair of uracil by Ung1 and consequently the formation of AP sites are the cause of the deleterious effect of the *dut1-1* mutation in WT and AP sites defective strains, whereas the persistence of uracil is not.

The *ung1 dut1-1* double mutant was then used to estimate the amount of uracil incorporated into genomic DNA. Genomic DNA was prepared from WT (FF18733), *ung1* (BG135), *dut1-1* (BG217) and *ung1 dut1-1* (BG234) strains. Afterwards, the purified DNA was incubated with an excess of the *E.coli* uracil DNA *N*-glycosylase, Ung, to release uracil residues, thus generating AP sites in DNA. The DNA was then incubated under alkaline condition to cleave the AP sites yielding single strand breaks. The DNA was further analyzed on an agarose alkaline gel to estimate the amount of breaks that corresponds to the amount of uracils in DNA. Figure 4A shows that the genomic DNA of the *ung1 dut1-1* strain was digested by Ung, in contrast to genomic DNA from WT, *dut1-1* or *ung1* strains. These last three strains exhibit no significant differences in their migration profiles (Figure 4A and data not shown). In the *ung1 dut1-1* double mutant, the average size of the digested DNA fragments is ~500 nucleotides (Figure 4B). These data allowed us to estimate that in the *ung1 dut1-1* strain, the level of substitution of uracil for thymine is ~1%. This result means that the steady-state level of uracils in this mutant is ~50 000 uracils per haploid genome of *S.cerevisiae*.

The *dut1-1* mutant is a spontaneous mutator that accumulates AT to CG transversions

Our data suggest that the *dut1-1* mutant has to cope with the formation of ~50 000 AP sites per generation. Since AP sites are mutagenic lesions, the *dut1-1* mutant was expected to be a spontaneous mutator. To measure mutation frequencies, we used a forward mutation assay using the *CAN1* gene as a reporter (34). The results show that the spontaneous frequency of canavanin-resistant (Can^R) mutants in the *dut1-1* strain is much more than in the WT strain (Table 3). To show that the mutagenesis was due to the *dut1-1* mutation, we introduced the plasmid p424GAL1 or p424GAL1-*DUT1* (6) in the *dut1-1* mutant. As expected, the presence of p424GAL1-*DUT1* in the *dut1-1* strain abolishes its spontaneous mutator phenotype whereas the control plasmid p424GAL1 does not (Table 3). If unrepaired AP sites are at the origin of the mutator phenotype of *dut1-1*, it should be greatly reduced in the *ung1 dut1-1* double mutant. Table 3 shows that the Can^R mutation frequency of the *ung1 dut1-1* double mutant is much less than that of the *dut1-1* single mutant, and similar to that of an *ung1* single mutant. These last results strongly suggest that the removal by Ung1 of dUMPs incorporated by DNA polymerases results in the formation of AP sites that are at the origin of the mutator phenotype of the *dut1-1* mutant.

The bypass of AP sites in the course of the translesion synthesis (TLS) process could explain the high mutation frequencies in the *dut1-1* strain. To check this hypothesis, we crossed the *dut1-1* mutant with a strain deleted for the *REV3* gene that encodes one of the subunit of the DNA polymerase ζ. The spontaneous mutation frequency of a *dut1-1 rev3* mutant (BG239) was decreased to a WT level (Table 3) confirming our hypothesis. To get insight into the molecular mechanism of the *dut1-1* mutagenesis, we decided to sequence Can^R mutations in WT, *dut1-1*, *ung1* and *dut1-1 ung1* mutants (Table 4). The results show that Can^R mutation spectra in WT, *ung1* and *ung1 dut1-1* strains are dominated by single base pair substitutions at G:C pairs (64, 76 and 73%, respectively) but there are no hot spots of mutagenesis. The mutation spectra of the *ung1* and *ung1 dut1-1* mutants are characterized by an important increase of GC to AT transitions compared with WT as previously shown in *E.coli* and *S.cerevisiae* (40,41). In *ung1* and *ung1 dut1-1* mutants, cytosine deamination is thought to be responsible for GC to AT transitions (40). In

Table 4. Spectra of Can^R mutations

Genotype	Mutation	Occurrence	Mutation Frequency[a] (10^{-7})
Wild type	GC to TA	11/61 (18%)	1.4
	GC to CG	8/61 (13%)	1.0
	GC to AT	20/61 (33%)	2.6
	AT to TA	2/61 (3%)	0.3
	AT to CG	7/61 (11%)	0.9
	AT to GC	3/61 (5%)	0.4
	(−1) deletion	5/61 (8%)	0.7
	insertions	2/61 (3%)	0.3
	complex[c]	3/61 (5%)	0.4
ung1	GC to TA	1/25 (4%)	1.5
	GC to AT	18/25 (72%)	27.4 [11][b]
	AT to TA	1/25 (4%)	1.5
	AT to CG	2/25 (8%)	3.0
	AT to GC	1/25 (4%)	1.5
	(−1) deletion	1/25 (4%)	1.5
	(+1) insertion	1/25 (4%)	1.5
dut1-1	GC to TA	1/54 (2%)	8.2
	GC to CG	1/54 (2%)	8.2
	GC to AT	1/54 (2%)	8.2
	AT to TA	4/54 (7%)	28.6 [95][b]
	AT to CG	33/54 (61%)	249.5 [277][b]
	AT to GC	5/54 (9%)	38.8 [97][b]
	(−1) deletion	4/54 (7%)	28.6
	deletion	1/54 (2%)	8.2
	(+1) insertion	1/54 (2%)	8.2
	insertion	2/54 (4%)	8.2
	complex	1/54 (2%)	16.4
ung1 dut1-1	GC to TA	4/30 (13%)	6.9
	GC to AT	18/30 (60%)	31.8 [12][b]
	AT to CG	2/30 (7%)	3.7
	AT to GC	2/30 (7%)	3.7
	(−1) deletion	3/30 (10%)	5.3
	(+1) insertion	1/30 (3%)	1.6

[a]Can^R mutation frequencies are the product of the proportion of a specific class of mutation and the total mutation frequency for each strain. In this experiments, values of mutation frequencies for WT, *ung1*, *dut1-1* and *dut1-1 ung1* were of: 8, 38, 409 and 53 per 10^7 viable cells, respectively.
[b]Number in brackets is the fold induction of a specific class of mutation relative to the WT.
[c]Complex mutations are the mutations that involve multiple molecular events.

contrast, the Can^R mutation spectrum in the *dut1-1* mutant was dominated by single base pair substitutions at A:T pairs (77% compared with 19% in the WT) with a majority of AT to CG transversions, 61% compared with 11% in the WT (Table 4). Indeed, the frequency of AT to CG is 277-fold higher in the *dut1-1* than in the WT. Furthermore, AT to TA transversions and AT to GC transitions are also enhanced compared to WT, 95- and 97-fold, respectively (Table 4). Therefore, Can^R mutation spectra in the *dut1-1* strain strongly suggests that dCMP (deoxyribocytidine monophosphate) is preferentially incorporated opposite AP sites in genomic DNA, whereas dGMP (deoxyriboguanosine monophosphate) and dTMP are incorporated at a lower efficiency.

The deletion of *DUT1* and the other face of uracil toxicity

The lack of a major growth problem exhibited by the *ung1 dut1-1* double mutant demonstrates that yeast can survive with moderate amounts of uracil substituted for thymine (~1%) in DNA. In the *dut1-1* mutant, the dUTPase activity is presumably sufficient *in vivo* to maintain a reasonably low dUTP pool. To dramatically increase the amount of uracil into DNA, we

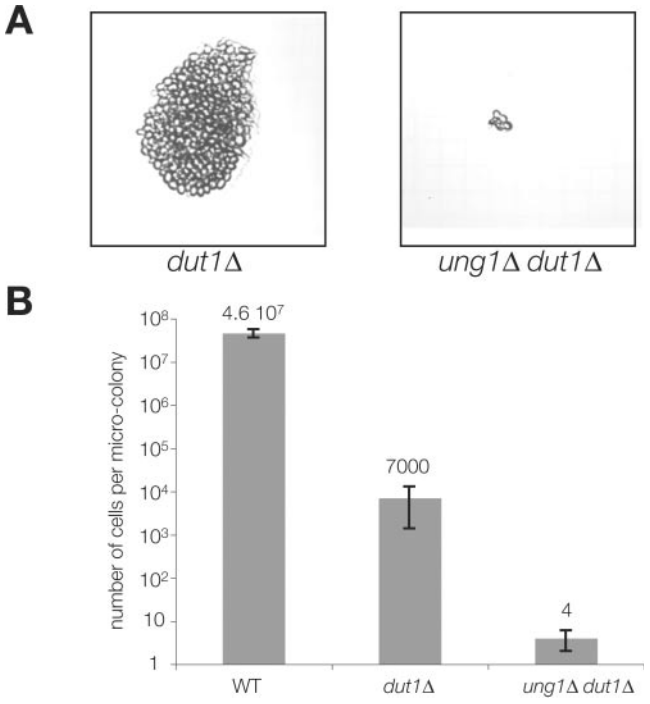

Figure 5. Inactivation of the *UNG1* gene aggravates the *dut1*Δ phenotype. The diploid strain BG235 heterozygous for *dut1*Δ/*DUT1* and *ung1*Δ/*UNG1* was sporulated and dissected on YPD at 30C. (**A**) 4 days after dissection, pictures of micro-colonies of *dut1*Δ and *ung1*Δ *dut1*Δ were taken. (**B**) The average number of cells in each microcolony is indicated and corresponds to at least five micro-colonies of two independent crosses, it was determined as previously described (2).

decided to delete the *DUT1* gene in both Ung1-proficient and Ung1-deficient backgrounds. To construct these strains, we sporulated a diploid strain heterozygote for *DUT1*/*dut1*Δ and *UNG1*/*ung1*Δ (BG235). After dissection, tetrad analysis was performed to identify the various genotypes. Our results show that the deletion of the *DUT1* gene causes cell death as already described (19) in presence or absence of the *UNG1* gene (Figure 5). Four days after dissection, we looked at the size of the micro-colonies formed by *dut1*Δ and *ung1 dut1*Δ mutants. The micro-colonies formed by the *dut1*Δ single mutant are composed of an average of 7000 cells, indicating a relatively slow process of cell death (Figure 5A, left panel and Figure 5B). In contrast, the micro-colonies of the *ung1 dut1*Δ double mutant were composed only of 2–4 cells (Figure 5A, right panel and Figure 5B). This result strongly suggests that very high amount of uracil in DNA is very deleterious to cells, even more toxic than AP sites generated via its excision by Ung1. These last results suggest that uracils in DNA can be toxic by two different modes depending on the amount present.

DISCUSSION

The aim of our research is to investigate the formation, the repair and the biological consequences of endogenous DNA damage in eukaryotic cells (1,5,42). Our recent data revealed that the burden of endogenous AP sites causes cell death in the absence of Apn1, Apn2 and Rad1-Rad10 (2). A critical source

of endogenous AP sites in DNA is the removal by an uracil DNA *N*-glycosylase of uracil residues that come from the use of dUTPs by DNA polymerases (6). In the present study, we developed a co-lethal screen to identify the cellular function(s) that can influence the formation of AP sites under physiological growth conditions. A mutant that carries a point mutation in the *DUT1* gene encoding the dUTPase in *S.cerevisiae* was isolated and named *dut1-1*. The point mutation is a single base substitution (G244A) that results in a single amino acid substitution (Gly82Ser) in the Dut1 protein. Sequences alignment reveals that this glycine is conserved in all Dut1 proteins from viral, bacterial or eukaryotic origin (38). Gly82 is localized in motif III of the five dUTPase motifs nearby Asp85 that is essential for Dut1 catalytic activity suggesting that the Dut1-G82S protein has a decreased dUTPase activity (38). In fact, the dUTPase activity in the *dut1-1* mutant is strongly diminished *in vitro*, it represents <10% of the dUTPase activity of a WT strain. Therefore, *dut1-1* is a viable allele of *DUT1* with a decreased dUTPase activity that allowed us, for the first time, to investigate the biological functions of the Dut1 protein in eukaryotes.

The *dut1-1* mutant exhibits increased division time, abnormal FACS profil and high levels of large budded cells with nucleus at the bud neck suggesting cell cycle arrest at G2/M transition. These data and our previous data (6) are consistent with the persistence of unrepaired DNA damage triggering checkpoint responses (43). Growth defects associated with the *dut1-1* allele most probably rely on a low dUTPase activity and consequently on a high dUTP pool. Since DNA polymerases do not differentiate dUTP from dTTP, the incorporation of dUMP into DNA must be significantly higher in a *dut1-1* mutant than in a WT strain, yielding an excess of U:A pairs in DNA (Figure 1). Although well-tolerated by itself, U:A pair becomes highly toxic after the excision of uracil by the uracil DNA *N*-glycosylase (Ung1) yielding an equivalent number of AP sites in DNA. The involvment of uracil and AP sites in the deleterious impact of the *dut1-1* allele is supported by the two following observations. First, the inactivation of the *UNG1* gene alleviates the deleterious effects associated with the *dut1-1* allele. Second, inactivation of both AP endonucleases Apn1 and Apn2 results in a severe aggravation of the phenotypes associated with *dut1-1*. Ultimately, this model implies the incorporation of a large number of dUMP in the genomic DNA of *dut1-1* and *ung1 dut1-1* strains. Indeed, the measurement of the steady-state level of uracil in DNA in the *ung1 dut1-1* double mutant reveals that the level of replacement of thymine by uracil in the genomic DNA is ~1%, which translates to ~50 000 uracil residues per genome. Therefore, the formation of ~50 000 AP sites per round of replication can explain growth defects of the *dut1-1* strains. It also explains the aggravation of the phenotypes of the *dut1-1* mutant when associated with defects in AP site repair such as in *apn1 apn2 dut1-1*, *apn1 rad14 dut1-1* and *apn1 apn2 rad14 dut1-1* mutants. These results are in agreement with a model that suggests that endogenous AP sites coming from the repair of uracils provoke the accumulation of 3′-blocked SSBs that are ultimately converted into double strand breaks leading to cell death after prolonged G2/M cell cycle arrest (1,2).

On the other hand, it is important to note that despite growth defects, the *dut1-1* single mutant remains viable. Therefore, yeast cells can cope with the formation of ~50 000 AP sites per genome per generation. This result points to a very high efficiency of DNA repair processes involved in the elimination of AP sites and primarily the AP endonuclease Apn1 that is part of the base excision repair pathway (1). Importantly, in the *dut1-1* mutant, the vast majority of AP sites are formed after the passage of the replication forks, therefore they are not blocks for DNA polymerases. In mouse cells, the nuclear Ung2 protein physically interacts with PCNA and RPA indicating a specialized role in counteracting U:A base pairs formed by the use of dUTP during DNA synthesis (44). Therefore, the post-replication mode of formation and repair of *dut1-1*-mediated AP sites can explain how cells can cope with such a high level of toxic DNA damage. However, a fraction of AP sites can form ahead of replication forks by incorporation of dUMP residues in DNA repair patches such as those resulting from the NER and the BER of endogenous DNA damages. Finally, a fraction of dUMPs or AP sites could be left unrepaired until the next round of replication.

In any case, the mutator phenotype of the *dut1-1* mutant and its suppression in Ung1- or Rev3-deficient cells strongly suggets that a fraction of AP sites are subject to the TLS pathway (45). Therefore, the *dut1-1* mutant can be used to investigate the mutagenic potential of AP sites *in vivo* in a chromosomal gene such as *CAN1*. The Can^R mutation spectrum shows a strong increase in mutations at A:T pairs consistent with the TLS of AP sites resulting from the excision of uracil at U:A pairs in DNA. Most of the mutations observed are tranversions AT to CG suggesting that the nucleotide incorporated in front of an AP site *in vivo* is a cytosine. The results also indicate that guanine and thymine are incorporated opposite AP sites, but at a lower rate. It has been proposed that during TLS of AP sites, the replicative DNA polymerase δ incorporates preferentially a dAMP opposite an AP site in MMS-treated cells. This DNA polymerase is then exchanged with the DNA polymerase ζ that subsequently extends from the inserted nucleotide (45). However, other studies report a preferential incorporation of dCMP opposite AP sites (46–49). In the first study, the incorporation of a cytosine cannot be observed since, after MMS treatment, the majority of AP sites come from the repair of methylated guanines. In the case of the *dut1-1* mutant, the incorporation of an adenine in front of an AP site that comes from the repair of an uracil should not be mutagenic and therefore cannot be investigated. However, it should be noted that the mutation spectrum of a *dut-1* mutant in *E.coli* presents an excess of base pair substitutions at G:C pairs and the AT to CG event is not represented (17). A potential explanation for these differences in mutation spectra between *E.coli dut-1* and *S.cerevisiae dut1-1* may rest on the preferential incorporation of adenine opposite an AP site (A-rule) in *E.coli* (17) and which may not exist in *S.cerevisiae*. The *dut1-1* mutant allowed us to investigate the mutagenic impact of AP sites on a chromosomal gene in undamaged yeast cells. Our data are in favor of the preferential incorpotation of dCMP opposite AP sites (C-rule) in WT strains and is in agreement with other studies (46–49). However, it seems that the nucleotide incorporated depends on the DNA polymerase used or/and on the damage that is at the origin of the AP site or/and the type of AP site itself (47,48).

The dUTPase activity is essential in *E.coli* and *S.cerevisiae* (12,19). In yeast, the *dut1*Δ mutant is lethal and can only form

micro-colonies of ~7000 cells. Previous work has suggested two major possibilities to explain the inviability of cells harboring a deletion of the dUTPase encoding gene (18). First, extensive excision repair of uracil-containing DNA could be lethal because of DNA fragmentation. Second, a high level of uracil in DNA may interfere with the binding to DNA of specific proteins required for the transcription of essential genes (50). The first possibility can explain the lethality of a *dut1*Δ and the extreme sickness of an *apn1 apn2 dut1-1* strain. The second possibility can explain why *ung1 dut1*Δ cells can only form very small micro-colonies of about four cells. In the *ung1 dut1*Δ double mutant, an excessive substitution, in one round of replication, of thymine by uracil in promoter regions can alter the expression of essential genes. The level of uracil substitution required to trigger an alteration of gene expression is unknown. Our data show that ~1% uracil substitution is tolerated by yeast cells. The characterization of the *dut1-1* strain of *S.cerevisiae* demonstrates that a critical threat to DNA in dividing cells is due to DNA metabolism by itself, not only its byproducts, since dUTP is a physiological intermediate in the course of dTTP biosynthesis in all organisms. Indeed, the process by which living organisms make most of its dTMP from triphosphate precursors is metabolically wasteful and entails the production of a harmful intermediate, dUTP. Perhaps dUTP is a relic of evolution, perhaps some uracil incorporation is desired because it promotes recombination, mutation or signalization and is thus beneficial to the cell population.

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