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Identification and characterization of *CRT10* as a novel regulator of *Saccharomyces cerevisiae* ribonucleotide reductase genes

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ABSTRACT

The *CRT10* gene was identified through screening of the *Saccharomyces cerevisiae* deletion library for hydroxyurea (HU) resistance. *CRT10* encodes a putative 957 amino acid, 110 kDa protein with a leucine repeat and a WD40 repeat near the N-terminus. Deletion of *CRT10* resulted in an enhanced resistance to HU reminiscent of the inactivation of two other ribonucleotide reductase (Rnr) suppressors, *CRT1* and *SML1*, which regulate Rnr activity at transcriptional and translational levels, respectively. Epistatic analysis indicates that *CRT10* belongs to the *CRT1* pathway but not the *SML1* pathway. Indeed, deletion of *CRT10* enhanced the survival of the *mec1* null mutant and increased basal level and DNA damage-induced expression of *RNR2* and *RNR3*, suggesting that Crt10 regulates *RNR* genes at the transcriptional level. Furthermore, the *dun1* mutation is epistatic to *crt10* with respect to both HU sensitivity and *RNR* gene expression. Interestingly, the expression of *CRT10* itself is induced by DNA damaging agents and this induction requires *DUN1*, suggesting that *CRT10* plays a role in cellular response to DNA damage and replication blocks. The *CRT10* function appears to be achieved by positive regulation of the *CRT1* transcript level, indicating that *CRT10* is a component of the regulatory circuit.

INTRODUCTION

Ribonucleotide reductase (Rnr) catalyzes the rate-limiting steps in dNTP synthesis. Three classes of Rnr have been identified (1). Class I enzymes, which are found in all eukaryotes and some prokaryotes, consist of an $\alpha_2\beta_2$ tetramer with two large (α) and two small (β) subunits. The α subunit possesses binding sites for substrate and allosteric effectors, and the β subunit contains a binuclear iron complex that interacts with a specific tyrosine residue to form a tyrosyl free radical and is essential for the Rnr activity (2,3). In the budding yeast *Saccharomyces cerevisiae*, the large Rnr subunit is encoded by two highly homologous genes, *RNR1* and *RNR3* (4). *RNR1* is an essential gene, whereas *RNR3* is nonessential. *RNR1* transcription is tightly regulated during the cell cycle and moderately induced by DNA damage, whereas *RNR3* is barely transcribed under normal conditions but is highly inducible by DNA damage, increasing up to 100-fold (4). The small Rnr subunit is encoded by *RNR2* and *RNR4*, both of which are essential and DNA damage inducible (5–7), although *RNR4* null mutants in some yeast strains appear to be viable (8).

The tight regulation of Rnr during the cell cycle and by DNA damage is thought to be crucial for the maintenance of balanced dNTP pools for high-fidelity DNA replication and repair (9,10). Failure to provide a sufficient and balanced dNTP pool may cause misincorporation of dNTPs into DNA, which in turn results in genetic abnormalities and cell death (11). The regulation of Rnr involves multiple mechanisms in budding yeast, including transcriptional regulation (12), protein (13) and allosteric (11,14) inhibition and subcellular localization (15). The DNA damage-induced transcriptional activation is mediated by the cell cycle checkpoint genes. The stalling of the replication fork or DNA damage triggers a DNA damage checkpoint pathway composed of the protein kinase cascade Mec1, Rad53 and Dun1 (16). Activated Dun1 phosphorylates a Crt1 repressor, and hyper-phosphorylated Crt1 no longer binds the X-box sequence found in the promoters of *RNR* genes, resulting in transcriptional derepression (17).

A second mechanism is Sml1-dependent; Sml1 inhibits the yeast Rnr activity by binding its large subunit (18–20). Activated Sml1 levels decrease at S phase and after DNA damage, resulting in derepression of Rnr activity (13). The inactivation of Sml1 is caused by post-transcriptional regulation and also

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requires Mec1-Rad53-Dun1-dependent phosphorylation (13,21), which again testifies to the need for tight Rnr regulation. The tight regulation of Rnr activity appears to be true for other organisms, such as fission yeast (22), indicating that such regulations are evolutionarily conserved.

It is anticipated that additional genes and/or mechanisms may be involved in the regulation of Rnr activities. To investigate this possibility, we utilized the powerful budding yeast genetic system to identify such genes, and report here the identification of a novel gene, *CRT10*, whose mutation enhances hydroxyurea (HU) resistance. Genetic characterization indicates that *CRT10* is involved in the transcriptional regulation of *RNR* genes.

MATERIALS AND METHODS

S.cerevisiae strains, cell culture and transformation

The yeast strains used in this study are listed in Table 1. Yeast cells were cultured at 30°C either in a YPD rich medium or in a synthetic dextrose (SD) medium supplemented with amino acids and bases (23). Yeast cell transformation was performed by using a dimethyl sulfoxide (DMSO)-enhanced method as described (24). For targeted gene integration, plasmid DNA was digested with restriction enzymes and the DNA was precipitated prior to transformation.

Screening of yeast deletion library

The yeast haploid deletion library was created by the *Saccharomyces* Genome Deletion Project consortium and purchased from Research Genetics (Invitrogen, Carlsbad, CA). The deletion mutants were replicated on to YPD and YPD + 80 mM HU. Plates were incubated at 30°C for 3 days before evaluation.

Cell killing by DNA-damaging agents

HU and methyl methanesulfonate (MMS) were purchased from Sigma–Aldrich (St. Louis, MO). Log phase yeast cells were diluted to 1×10^7 cells/ml, and 10-fold serial dilutions were made. Aliquots of 10 μl of diluted cells were spotted on the appropriate plates, and incubated at 30°C for 3 days.

Table 1. *Saccharomyces cerevisiae* strains

Strain	Genotype	Source
BY4741	*MAT**a** his3 leu2 met15 ura3*	Invitrogen
WXY1152	BY4741 with *crt10Δ::KanMX*	Invitrogen
WXY1153	BY4741 with *crt1Δ::KanMX*	Invitrogen
WXY1154	BY4741 with *crt1Δ::KanMX crt10Δ::LEU2*	This study
WXY1155	BY4741 with *dun1Δ::KanMX*	Invitrogen
WXY1156	BY4741 with *dun1Δ::KanMX crt10Δ::LEU2*	This study
HK578-10A[a]	*MAT**a** ade2-1 can1-100 his3-11,15 leu2-3112 trp-1-1 ura3-1*	H. Klein
WXY1157[a]	HK578-10A with *crt10Δ::LEU2*	This study
HK578-10D[a]	*MATα ade2-1 can1-100 his3-11,15 leu2-3112 trp-1-1 ura3-1*	H. Klein
WXY1158[a]	HK578-10D with *crt10Δ::LEU2*	This study
U952-3B[a]	*MAT**a** sml1Δ::HIS3*	R. Rothstein
U953-61A[a]	*MAT**a** mec1Δ::TRP1 sml1Δ::HIS3*	R. Rothstein
WXY1159[a]	U952-3B with *crt10Δ::LEU2*	This study

[a]These strains are isogenic to W303 but contain a wild-type *RAD5* gene.

Plasmids and plasmid construction

Plasmid manipulation was performed using enzymes purchased from Invitrogen and New England Biolabs (Beverly, MA) as recommended by the manufacturers.

Plasmids pZZ2 and pZZ18, containing the *RNR3-lacZ* and *RNR2-lacZ* reporter genes, respectively (25), were kindly provided by Dr S. Elledge (Harvard Medical School, Boston, MA). To construct *crt10Δ::LEU2* disruption cassettes, the 2.8 kb *CRT10* open reading frame was PCR-amplified from genomic DNA with primer CRT10-1: 5′-CCGGAATT-CATGCCCCCTCAGATTCCCAATG-3′, and CRT10-2: 5′-CGGGTCGACCTATTGTTGAGTTGTTCCATG. The PCR product was cloned into pBluescript SK (Stratagene, La Jolla, CA) to form pBS-CRT10. The resulting plasmid pBS-CRT10 was digested by HpaI and MscI to remove the fragment encoding 60–871 amino acid, and then ligated with a BglII linker to create plasmid pBS-crt10Δ. A *LEU2* marker was inserted into the plasmid to form pcrt10Δ::LEU2. For *CRT10* disruption, pcrt10Δ::LEU2 was digested by AvaI and NcoI prior to yeast transformation.

Yeast tetrad analysis

For tetrad analysis, parental haploid strains U953-61A and WX1158 were mated on SD-Trp-His-Leu plates and transferred into sporulation medium. After 3 days incubation at room temperature, the spores were dissected and incubated for 3 days prior to phenotyping by replica plating to YPD and SD medium containing appropriate combinations of amino acids. To minimize the possibility of dissection of false tetrads, digestion of asci was carried out by incubating with NEE-154 glusulase (Dupont Company, Wilmington, DE, USA) at room temperature for 10 min immediately before dissecting. The tetrads were dissected with a Singer MSM micromanipulator (Singer Instrument Co., Somerset, England).

RNA extraction, northern hybridization and real-time PCR

Yeast cells with or without treatment were harvested from early log phase culture (2×10^7 cells/ml). Total RNA was prepared using an RNeasy midi Kit (Qiagen, Valencia, CA). Northern blot analysis was performed as described previously (26). The DNA probe was labeled with [α-^{32}P]dCTP using a Random Primer Labeling kit from Invitrogen. The *CRT10* PCR product containing the entire open reading frame and the 1.6 kb *ACT1* fragment from pAA93 (a gift from Dr F. Sherman, Rochester University, NY) were used as probes.

For real-time PCR, the extracted RNA was treated with a DNA-free™ Kit from Ambion (Austin, TX) to remove contaminating DNA. The treated RNA was used as template to perform reverse transcription through the Thermoscript™ RT–PCR system (Invitrogen). Real-time PCR was carried out and analyzed by the MiniOpticon™ real-time PCR system (BioRad, Hercules, CA). Primers used in the real-time PCR were: ACT1-1 (5′-TGGCCGGTAGAGATTTGACTGACT-3′); ACT1-2 (5′-AGAAGCCAAGATAGAACCACCAAT-3′); TUP1-1 (5′-CCACCACGTCGACGGATAACAATA-3′);

TUP1-2 (5′-CTCGGAATCCCAAACTCTCACAGC-3′); SSN6-1 (5′-GCCCAAGCTCCCCAACC-3′); SSN6-2 (5′-CTGTGCGCCAATTACTGAAGG-3′); CRT1-1 (5′-GGTCG-CCCGTTAAACAGAGTA-3′) and CRT1-2 (CGTGGGCGA-TATAGAGTTAGAGT-3′); MAG1-R1 (5′-GCGGTGCATT-TCCTGATTA-3′) and MAG1-R2 (5′-TCGCGAGCCTC-CAAAGTAT-3′); and RNR3-R1 (5′-GCCTCCGCTGCTA-TTCAA-3′) and RNR3-R2 (5′-CAGATGCCGCCTTTT-GTT-3). The relative transcript level of each treatment was determined by a method and formula as described (27).

β-Galactosidase (β-gal) assay

The β-gal assay was performed as described previously (26,28). Briefly, 0.5 ml of overnight yeast culture was used to inoculate 2.5 ml of fresh SD selective medium and incubation was continued for another 2 h. At this point, chemicals were added at the concentration indicated and cells were incubated for another 4 h. One ml of cell suspension was used for determining cell titer at OD_{600nm}, and the remaining cells were used for the β-gal assay. The β-gal activity is expressed in Miller units (29).

RESULTS

Identification of *CRT10*

HU is a potent inhibitor of Rnr, leading to depleted dNTP pools, the subsequent stalling of the replication forks and S phase cell cycle arrest (2,30). In order to identify *S.cerevisiae* genes whose mutation alters cellular sensitivity to HU, we performed an HU resistance screen with the haploid yeast mutant library consisting of 4850 individual gene deletion strains. Among HU-resistant mutants, the *YOL063c* deletion mutant displayed significant resistance to HU and this gene has not been previously characterized. *YOL063c* encodes a putative 957 amino acid, 109 kDa protein and was designated as *CRT10*, after the nine previously described putative *CRT* (*c*onstitutive *R*NR *t*ranscription) regulator genes (25).

The predicted Crt10 contains leucine repeats at residues 105–145 with a sequence L-X_9-L-X_8-L-X_6-L-X_6-L-X_6-L, a putative transmembrane domain at residues 191–206, and one copy of the *Trp-Asp* (WD) repeat motif at residues 253–267 (31) (Figure 1). WD-repeat proteins are found in all eukaryotes and implicated in a wide range of crucial functions. These proteins typically contain 4–16 copies of the WD motif (32); however, only one WD repeat motif was found in Crt10.

A database search with the Crt10 protein sequence revealed several homologous sequences in other organisms. The closest homologs are found in members within the *Saccharomyces* family. In addition, a putative protein (ADR329Wp) in *Eremothecium gossypii* and *Candida albicans* hypothetical protein (CAG58307.1) show significant homology to Crt10; a hypothetic protein (SPBC27B12.05) from *Schizosaccharomyces pombe* also shows limited homology to Crt10 (data not shown). No polypeptide sequence in the worm, mouse or human genome database has significant similarity to Crt10, suggesting that Crt10 may be unique to lower eukaryotes, possibly within unicellular eukaryotic microorganisms.

Deletion of *CRT10* enhances survival of the *mec1*Δ mutant

The *crt10* mutant was originally isolated for its enhanced resistance to HU killing in a library screen. We compared the *crt10*Δ mutant to its isogenic wild-type strain BY4741 and found that it indeed displayed an enhanced resistance to HU (Figure 2A). In order to rule out the possibility that this *crt10* strain contains additional unknown mutation(s), we made a *crt10*Δ*::LEU2* deletion cassette and created a *crt10* null mutant in a different strain background. As shown in Figure 2B, targeted deletion of *CRT10* also resulted in a similar HU-resistant phenotype. Deletion of *CRT10* led to slightly enhanced resistance to MMS, but not to ultraviolet (UV) (data not shown), suggesting that Crt10 probably functions specifically in a pathway in response to either DNA replication arrest or an imbalance of the endogenous nucleotide pool.

To distinguish the above two possibilities, we took advantage of a cell cycle checkpoint mutant, *mec1*. It is known that the *mec1* null mutant is inviable; however, its inviability is due to the decreased expression of *RNR* genes rather than the loss of checkpoint functions. Hence, its viability can be rescued by deletion of either the Rnr inhibitor gene *SML1* (18) or the *RNR* repressor *CRT1* (17), or by overexpression of *RNR1* (33). We reasoned that if Crt10 acts upon Rnr expression/activity, deletion of *CRT10* may be able to rescue the *mec1*Δ inviability,

MPPQIPNENDDLFTRWLKSRAIIQRAVSTRECFDSEVFLASGGWNITNEIITLKKYYQLK 60
WPNSSCNSFHPKTVEFIKERLHNLEEHDSSWKIPNPAYSFKKAFLEDTKSAFSNLEPVWG 120
PSRLLNPAELLLPQDEKLLVQEIPLEFAPFQYTNRFAYGGLQFKNNLFVTYGSYSFLAAG 180
QCVEVHNFDI**LLNVSSLEICHALLPV**IIPDDGDVRNFRNSSYVKFKDTQFNSIPELCSIN 240
FMKICNFMHQDF***LLACGDNGIVYIWEI***NKVIKIFNKFTSDILGGKDNSRERYINVDPYMV 300
LRVEESCWSVDVIDINGIIYIAVGHNKPGVTVFAFDKDVKKERRYIRPLDLPSSHNVPCV 360
NFVPNSKDSVGYITLSYCSIFGNVVTVKLKEHDCTILTSFLDTQFFGDDLWTITPLTKKD 420
FAKVDNFELLNLNYQDGFKESMLYSICRDDFLLGYYCDNAYLSGNFGIGTLLNQFQVPVT 480
DLRLTSSAGIPDEVIPLRFTSFDRNYTTTGSIKYEYSREDFALILHAGDLDDMNDAVTKN 540
TSCEQHLHQWTFWEDSGYKHYRATERGFSKYKDIINTFPQLITPSGRNKTSQYQNTSGRK 600
ICEPSTYKLTDLENDIEDISREFNRSIRNLKMDKQRQLRTSKEFKSLSSVNHIPNIESGN 660
FLWYNTDAAADWRTLFGKDLNTVLKDPEICSLQLNSTEEDDVNSDPENEESGSSLTSFQR 720
RYRDTEQRAHLKSESQKSWGFHNYVRNVKRLLESAVPGSEDSPLGYQLSEMHDEFFFLTT 780
AHRLVLMKANPLIIISATHHEIFPLDGVVTCASKSLLQALNRINFVCHIKELNCIAVASQ 840
LGLISLLRLTEYRGIYSFRQEYILGWEVQDPVNPSPECRCNRNLFDAPMYGADGESSDTY 900
CGVCDVYFPMGDICGLDYTYASDSEELKRKGYATLYVASRGSLRAFKITTEHGTTQQ 957

Figure 1. The deduced *S.cerevisiae* Crt10/YOL063c amino acid sequence. The putative leucine repeat (underlined), transmembrane domain (boldface) and WD repeat (boldface and italicized) are indicated.

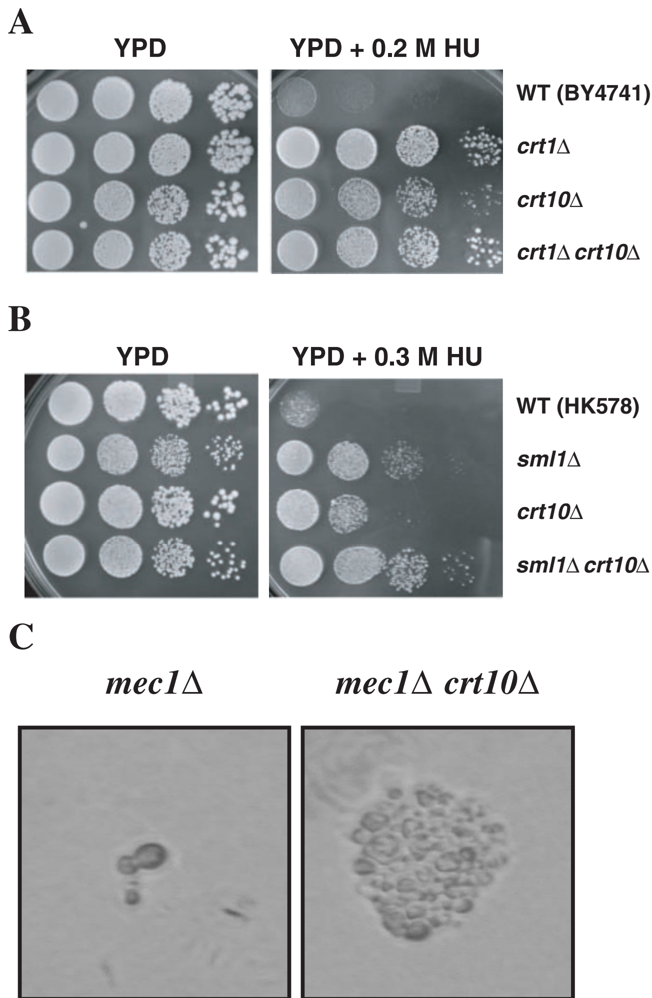

Figure 2. Genetic interaction of *CRT10* with *CRT1* (**A**), *SML1* (**B**) and *MEC1* (**C**). (A and B) Epistasis analysis. 10-fold serial dilutions of log-phase cultures were spotted on YPD plates and on YPD plates containing HU as described. Plates were incubated for 3 days at 30°C before photographing. Strains used: (A) BY4741 (WT), WX1152 (*crt10*Δ), WX1153 (*crt1*Δ) and WXY1154 (*crt1*Δ *crt10*Δ). (B) HK578-10A (WT), U952-3B (*sml1*Δ), WXY1157 (*crt10*Δ) and WXY1159 (*sml1*Δ *crt10*Δ). (C) The inviability of *mec1*Δ is partially rescued by deletion of *CRT10*. Tetrads resulting from a cross of WXY1158 (*MEC1 SML1 crt10*Δ) and U953-61A (*mec1*Δ *sml1*Δ *CRT10*) were dissected and the growth of each spore was followed by microscopic analysis. The representative picture was taken after 4 day incubation at 30°C.

whereas if it acts upon stalled replication fork, *CRT10* deletion should not be able to rescue *mec1*Δ. The *mec1*Δ *sml1*Δ double mutant was crossed to *crt10*Δ in an isogenic background. Haploid spores recovered from 40 tetrads were genotyped by replica plating to appropriate media. No viable *crt10 mec1* double mutant colonies were obtained. However, under the microscope, it was found that the *mec1*Δ mutant cells did not extend beyond two cell divisions, whereas the *crt10*Δ *mec1*Δ double mutant cells formed microcolonies containing up to several hundred cells (Figure 2C). This is in contrast to the *sml1 mec1* double mutant from the same experiment, which formed visible colonies (data not shown). Hence, deletion of *CRT10* appears to rescue *mec1*Δ cells from immediate death.

CRT10 belongs to the *CRT1* regulatory pathway

Since deletion of *CRT10* results in HU resistance and partially rescues the *mec1*Δ mutant, it is most likely involved in the regulation of Rnr activity. *CRT1* and *SML1* are two genes regulating Rnr by different mechanisms, the former at the transcriptional level (17) and the latter at the protein activity level (18). Indeed, we isolated both *crt1* and *sml1* during the initial mutant library screen. In order to ask if *CRT10* belongs to one of the two regulatory pathways, epistasis analysis was performed by creating *crt10*Δ *crt1*Δ and *crt10*Δ *sml1*Δ double mutants and comparing them to the corresponding single mutants with respect to HU resistance. The *crt10*Δ *crt1*Δ double mutant showed the same level of resistance to HU as the *crt1*Δ single mutant (Figure 2A), indicating that *CRT10* belongs to the same pathway as *CRT1*. In contrast, the phenotypic effect of *crt10*Δ appears to be additive with *sml1*Δ (Figure 2B), suggesting that *CRT10* does not belong to the same regulatory pathway as *SML1*.

The transcript level of *RNR* is elevated in *crt10*Δ mutants

Crt1 is an X-box DNA binding protein and represses the transcription of *RNR2*, *RNR3* and *RNR4* through recruitment of the corepressor complex Tup1-Ssn6; deletion of *CRT1* elevated the basal level expression of *RNR3* 25-fold (17). The above epistatic analysis predicts that deletion of *CRT10* may result in an elevated *RNR* gene expression as well. The β-gal activities of *RNR3-lacZ* and *RNR2-lacZ* transformants were measured in the wild-type and isogenic *crt10*Δ mutants with or without MMS or HU treatment. Indeed, the *RNR3-lacZ* and *RNR2-lacZ* levels were elevated about 2-fold in *crt10*Δ mutants compared to wild-type cells after treatment with DNA damaging agents (Figure 3). This result is consistent with a real-time PCR assay of the endogenous *RNR3* transcript (Table 2), suggesting that Crt10 functions as a transcriptional repressor to regulate *RNR2* and *RNR3* in budding yeast.

In order to further demonstrate that *CRT10* and *CRT1* belong to the same regulatory pathway, we measured the expression of *RNR3-lacZ* in wild-type, *crt1*Δ, *crt10*Δ single and the *crt1*Δ *crt10*Δ double mutants by β-gal assay. As shown in Table 3, the basal level of *RNR3-lacZ* was moderately elevated in the *crt10*Δ mutant and dramatically elevated in the *crt1*Δ mutant. Nevertheless, deletion of *CRT10* does not further enhance *RNR3-lacZ* expression in the *crt1*Δ mutant. The same effect holds true after MMS treatment. These results are consistent with the hypothesis that *CRT10* and *CRT1* function in the same pathway to regulate the transcription of *RNR* genes.

CRT10 functions downstream of *DUN1*

The observation that *crt1* is epistatic to *crt10* with respect to both HU resistance and *RNR* gene activity suggests that Crt1 most likely acts downstream of Crt10. The activity of Crt1 is regulated by its phosphorylation state, and the phosphorylation of Crt1 requires the protein kinase Dun1, although whether Dun1 directly phosphorylates Crt1 remains to be determined (17). In order to determine the genetic interaction between *CRT10* and *DUN1*, a *crt10*Δ *dun1*Δ double mutant was created and compared to its corresponding single mutants with respect to HU sensitivity. As seen in Figure 4A, whereas deletion of *DUN1* enhances HU sensitivity and deletion of *CRT10* results

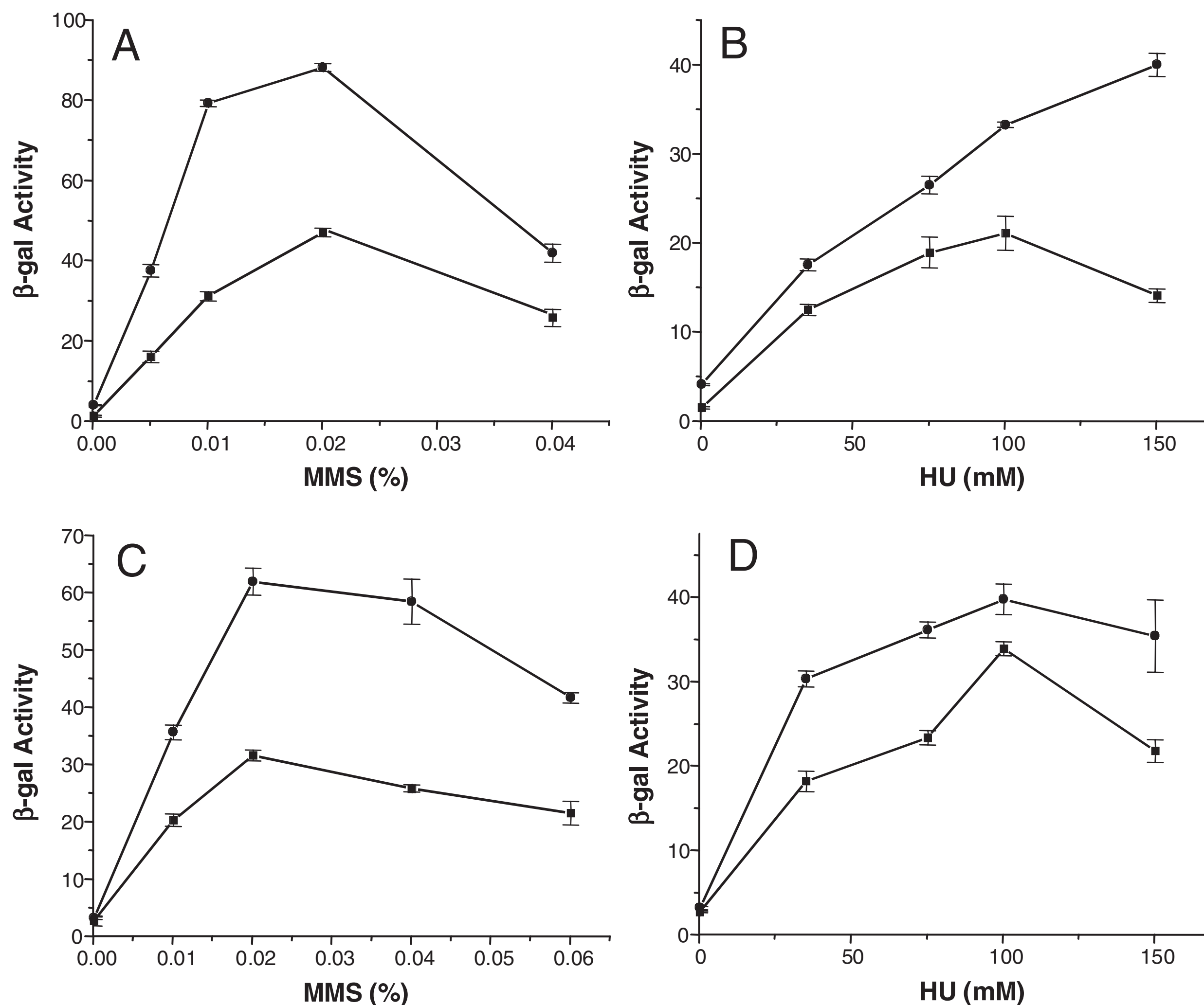

Figure 3. *RNR* gene expression in wild-type and *crt10*Δ cells. *RNR3-lacZ* (**A** and **B**) and *RNR2-lacZ* (**C** and **D**) expression was monitored after MMS (A,C) and HU (B,D) treatments. β-gal activity was determined as described in Materials and Methods. (filled square) BY4741 (wild type) and (filled circle) WXY1152 (*crt10*Δ) were transformed with either pZZ2 (pRNR3-lacZ) or pZZ18 (pRNR2-lacZ) and several independent transformants were picked for analysis. The results are the average of at least three independent experiments with standard deviations. β-gal activity is given in Miller units.

Table 2. Relative steady-state transcript level

Strain	Relative transcript level[a] HK578-10D (WT)		WXY1158 (*crt10*Δ)	
Treatment	−HU	+0.2 M HU	−HU	+0.2 M HU
RNR3	1	9.14	2.71	26.74
MAG1	1	2.32	1.27	2.19
TUP1	1	1.20	0.94	1.09
SSN6	1	0.99	1.02	0.99
CRT1	1	2.06	0.38	0.50

[a]Transcript levels were measured by real-time PCR with total mRNA from cells with or without treatment with 0.2 M HU for 1 h, and normalized to the *ACT1* transcript control. Untreated wild-type cells were used as a reference. Experimental variations due to PCR is negligible.

Table 3. β-gal activities of *RNR3-lacZ* in *crt1*Δ and *crt10*Δ mutants

Strain[a]	β-Galactosidase activity (Miller units)[b] −MMS	+0.02% MMS
BY4741	1.6 ± 0.12	48.9 ± 1.75
WXY1153 (*crt1*Δ)	72.5 ± 2.72	101.3 ± 3.51
WXY1152 (*crt10*Δ)	4.2 ± 0.32	85.5 ± 1.60
WXY1154 (*crt1*Δ *crt10*Δ)	73.8 ± 3.51	100.8 ± 2.74

[a]All strains were transformed with pZZ2 (*RNR3-lacZ*).
[b]β-gal activity was measured as described in Materials and Methods. Data represent the averages of at least three independent experiments with standard deviations.

in HU resistance, cells carrying both deletions display a phenotype indistinguishable from that of *dun1*Δ mutant. Similarly, *dun1* is epistatic to *crt10* with respect to *RNR3* expression, as deletion of *CRT10* did not alter the reduced *RNR3* induction in the *dun1* mutant (Figure 4B). These observations indicate that the HU resistance and increased *RNR* expression caused by *CRT10* deletion require functional Dun1.

Crt10 may act either upstream or downstream of Dun1. Dun1 is a multi-functional protein involved in gene regulation (21) as well as cell cycle checkpoints (34,35). Deletion of *DUN1* not only affects *RNR* gene induction, but also other

DNA damage-inducible gene expression (36). We reasoned that if Crt10 acts upstream of Dun1, its inactivation would alter all Dun1-mediated activities. If, as previously observed, Crt10 only affects a subset (i.e. *RNR*) of Dun1-mediated gene expression; inactivation of Crt10 should not affect other gene expression. For example, *MAG1* induction by DNA damage requires Dun1 (36); we found that its expression and induction was not altered by deletion of *CRT10* (Table 2), suggesting that indeed Crt10 acts downstream of Dun1 and is specific for *RNR* gene expression.

CRT10 is required for *CRT1* expression and induction

The above genetic analyses fit into a model that Crt10 functions as a positive regulator of Crt1 and/or its co-repressors Tup1-Ssn6. We thus measured the transcript levels of *CRT1*, *TUP1* and *SSN6* with or without HU treatment. As shown in Table 2, deletion of *CRT10* does not affect the transcript level of *TUP1* or *SSN6* regardless of HU treatment, but significantly reduced the basal level as well as HU-induced expression of *CRT1*. Hence, Crt10 appears to serve as a positive regulator of Crt1 at the transcriptional level.

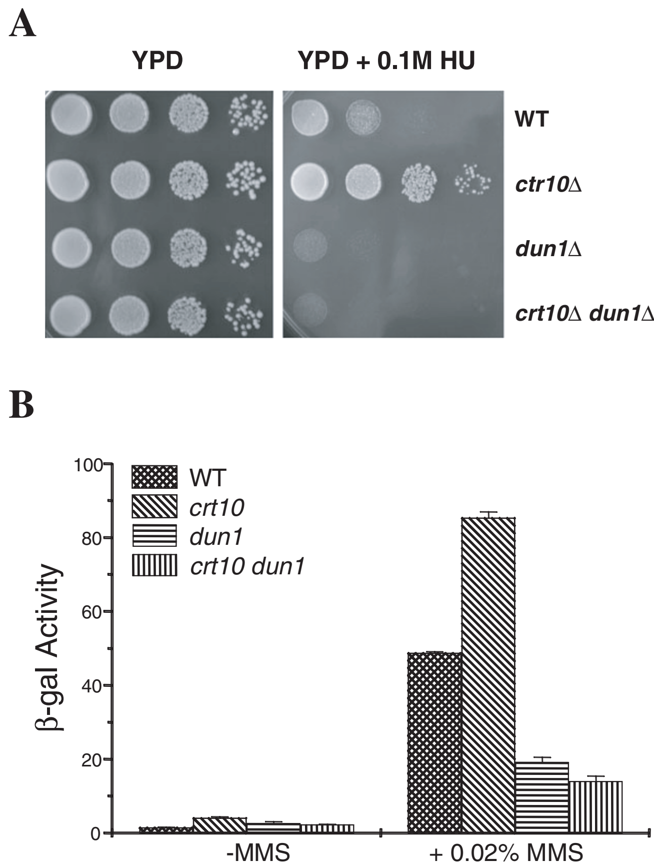

Figure 4. *DUN1* is epistatic to *CRT10*. (**A**) Deletion of *DUN1* abolishes the HU resistance caused by the *crt10* mutation. 10-fold serial dilutions were spotted on YPD plates and on YPD plates containing 0.1 M HU. Plates were incubated for 3 days at 30°C before photographing. Strains used: BY4741 (WT), WXY1152 (*crt10*Δ), WXY1155 (*dun1*Δ), WX1156 (*crt10*Δ *dun1*Δ). (**B**) The *CRT10* effect on *RNR3* expression is dependent on *DUN1*. *RNR3-lacZ* expression was monitored with or without MMS treatment and expressed in Miller units. The results are the average of at least three independent experiments with standard deviations.

Expression of *CRT10* is elevated in response to DNA damage and HU

Many genes involved in DNA metabolism (replication, repair and recombination) are induced after treatment with DNA damaging agents or replication blocking agents. In addition, regulatory genes, such as *CRT1* itself are up-regulated in response to DNA damage or HU treatment in a *DUN1*-dependent manner (17), indicative of an auto-regulatory circuit. We measured the *CRT10* transcript level under treated and untreated conditions and found that the *CRT10* transcript level is increased after treatment with MMS, HU and γ-rays (Figure 5A). Interestingly, there appear to be two transcripts with slightly different sizes; the treatments induce expression of both transcripts, but the higher molecular weight transcript is induced more dramatically than the lower molecular weight transcript. In order to address whether the transcriptional regulation of *CRT10* is dependent on other regulators in this pathway, such as Crt1 and Dun1, we compared the *CRT10* transcript levels in the wild-type and mutant backgrounds. The induction of *CRT10* requires *DUN1*, as the *dun1* mutation completely abolished *CRT10* induction, whereas deletion of *CRT1* has no effect on *CRT10* expression (Figure 5B).

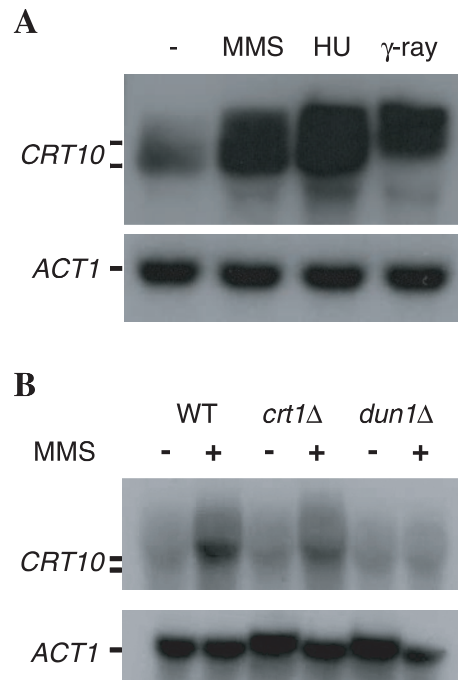

Figure 5. (**A**) *CRT10* expression in response to DNA damage and HU treatment. Log-phase wild-type HK578-10A cells were either untreated (lane 1) or treated with 0.3% MMS for 2 h (lane 2), 0.2 M HU for 2 h (lane 3) or exposed to 40 krad of γ radiation (lane 4). (**B**) *CRT10* induction is *DUN1*-dependent. Log-phase wild-type BY4741 and its derivatives WXY1153 (*crt1*Δ) and WXY1155 (*dun1*Δ) were either untreated (−) or treated with 0.1% MMS for 2 h (+). Northern hybridization was performed as described in Materials and Methods. The membranes were hybridized with *CRT10* (upper panel), stripped and then hybridized with *ACT1* (lower panel) as an internal control. Each lane contains 15 μg of total RNA.

DISCUSSION

We report here the isolation and initial characterization of *CRT10* as a novel yeast gene involved in the transcriptional regulation of *RNR* genes. Rnr catalyzes a rate-limiting step in the production of dNTPs, whose levels are critical to many cellular functions (10). Imbalanced or insufficient dNTP pools lead to enhanced misincorporation, high mutation frequencies and impaired DNA repair (11). Due to its vital importance to cellular physiology, it is not surprising that Rnr is tightly regulated via multiple mechanisms and at different stages. Our results suggest that Crt10 is a newly discovered negative regulator of *RNR* genes and acts at the transcriptional level. First, deletion of *CRT10* results in enhanced cellular resistance to HU, an Rnr inhibitor. Second, deletion of *CRT10* enhances the survival of the *mec1* null mutant, reminiscent of other suppressors of *mec1* and *rad53* inviability, all of which lead to increased Rnr activities (17,18,20,33). Third, deletion of *CRT10* in wild-type cells results in an increased expression of *RNR* genes coding for both large and small Rnr subunits, in the presence and absence of DNA damage, which provides underlying mechanisms of HU resistance. Results obtained from epistasis analyses suggest that Crt10 functions downstream of Dun1 and probably upstream of or together with Crt1 (Figure 6). Nevertheless, both *dun1* and *crt1* are epistatic to *crt10*, suggesting that Crt10 is probably a regulatory component in the Dun1-Crt1 signal transduction pathway leading to the control of *RNR* gene expression. Finally, our observation that deletion of *CRT10* reduces *CRT1* expression and abolishes the DNA damage induction of *CRT1* provides direct evidence that *CRT10* functions through positive regulation of *CRT1* expression.

The physiological significance of *RNR* suppression by *CRT10* is presently unclear; however, one interesting observation through this study is that *CRT10* itself is induced after DNA damage and HU treatment, suggesting that Crt10 plays a critical role in responding to replication blocks. Several pieces of evidence indicate that Crt10 achieves this objective through delicate regulation of the endogenous dNTP pool, as illustrated in Figure 6. Firstly, Crt10 acts as a negative regulator to counterbalance the dNTP pool. In the presence of replication blocks (DNA damage) or with an exhausted dNTP pool (HU treatment to inhibit Rnr activity), all four *RNR* genes are upregulated and Sml1 activity is inhibited, leading to enhanced dNTP production. The increased Crt10 activity may be required to bring Rnr activity back to a normal level once order is restored. In this respect, it is of great interest to notice that the optimal dose required to induce *CRT10* is higher than that required to induce *RNR* genes (37), which is consistent with the notion that *CRT10* induction may lag behind that of *RNR* genes. Secondly, like *CRT1* (17), the induction of *CRT10* itself depends on *DUN1*, suggesting that Crt10 functions downstream of Dun1 and forms another component of the autoregulatory circuit. However, the effect of *CRT10* deletion on *RNR* gene expression is much less than that of *CRT1* deletion and, unlike *CRT1*, the *CRT10* promoter does not contain the X-box sequence recognized by Crt1 (17). This is not unprecedented since DNA damage induction of several other genes also requires *DUN1* in the absence of the X-box sequence (36). Finally, in addition to its roles in modulating and maintaining an optimal dNTP pool under stress conditions, *CRT10* appears to be required for optimal growth in the absence of exogenous DNA damage/replication blocks, since a recent genetic footprinting study (38) showed that deletion of *CRT10* causes an apparently severe growth defect in rich medium after 20 generations, in minimal medium as well as medium containing NaCl. These observations imply that vigorous modulation of the endogenous dNTP pool by *CRT10* is critical to achieve optimal cell growth, possibly by maintaining proper DNA synthesis and cell division. Alternatively, *CRT10* may play roles in optimizing cell growth by a mechanism other than affecting Rnr activity.

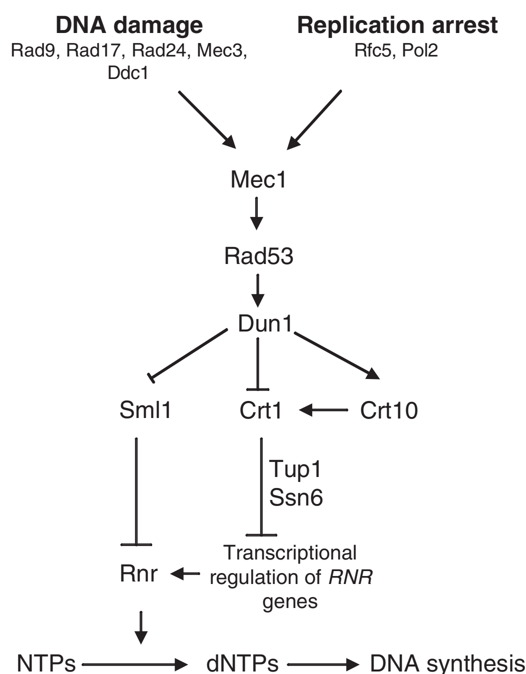

Figure 6. A proposed model for *CRT10* in Rnr regulation. Note that the *CRT10* functions and regulation are based on its genetic data instead of protein activity. Note that Rnr catalyzes the rate-limiting step in dNTP production.

Despite the strong genetic evidence that Crt10 is involved in the transcriptional regulation of *RNR* genes and *CRT1*, its biochemical activity remains obscure. The leucine repeats and a single WD motif suggest that Crt10 may interact with other protein(s), although to date no such proteins have been identified through systematic studies. It does not contain a domain/motif indicative of its catalytic function; however, the protein is apparently conserved and widespread within unicellular lower eukaryotes. Future investigations will attempt to uncover biological and biochemical functions of Crt10.

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