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 mecl 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 ctt10 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 α2β2 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 Smn1-dependent; Smn1 inhibits the yeast Rnr activity by binding its large subunit (18–20). Activated Smn1 levels decrease at S phase and after DNA damage, resulting in derepression of Rnr activity (13). The inactivation of Smn1 is caused by post-transcriptional regulation and also
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

Saccharomyces 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 the yeast haploid deletion library was created by the Screening of yeast deletion library

was digested with restriction enzymes and the DNA was precipitated prior to transformation. The treated RNA was used as template for 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'-AGGGAGAGATCAGAGAACCCAC-3'); ACT1-2 (5'-GGCCGCGATGATGATTCTTTGC-3'); TUP1-1 (5'-GGGGGACGACGCGGATAAGC-3'); TUP1-2 (5'-GGGGGACGACGCGGATAAGC-3'); TUP1-3 (5'-GGGGGACGACGCGGATAAGC-3'); TUP1-4 (5'-GGGGGACGACGCGGATAAGC-3').

Table 1. Saccharomyces cerevisiae strains

| Strain     | Genotype          | Source          |
|------------|-------------------|-----------------|
| BY4741     | MATa his3 leu2 met15 ura3 | Invitrogen      |
| WXY1152    | BY4741 with crt10a::KanMX | Invitrogen      |
| WXY1153    | BY4741 with cr1a::KanMX | Invitrogen      |
| WXY1154    | BY4741 with crt1a::KanMX cr10a::LEU2 | This study      |
| WXY1155    | BY4741 with dun1a::KanMX | Invitrogen      |
| WXY1156    | BY4741 with dun1a::KanMX cr10a::LEU2 | This study      |
| HK578-10Aa | MATa ade2-1 can1-100 his3-11,15 leu2-3112 trp-1-1 ura3-1 | H. Klein        |
| WXY1157a   | HK578-10Aa with cr10a::LEU2 | This study      |
| HK578-10Dd | MATa ade2-1 can1-100 his3-11,15 leu2-3112 trp-1-1 ura3-1 | H. Klein        |
| WXY1158b   | HK578-10Dd with cr10a::LEU2 | This study      |
| U952-3Ba   | MATa schl::HIS3 | R. Rothstein    |
| U953-61Aa  | MATa mec1::TRP1 schl::HIS3 | R. Rothstein    |
| WXY1159b   | U952-3B with cr10a::LEU2 | This study      |

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

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 ascii 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 × 10⁷ 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 [γ-³²P]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'-TTGCGCTGTTAGGATTTGCAGCT-3'); ACT1-2 (5'-AGGAGGAGATCAGAGAACCCAC-3'); TUP1-1 (5'-GGGGGACGACGCGGATAAGC-3');
TUP1-2 (5’-CTCGGAATCCAAAATCTGTCAACG-3’); SSN6-2 (5’-GGCGCAAGCTCCCAAAC-3’); SSN6-2 (5’-TTGTCGCAATCTGAAG-3’); CRT1-1 (5’-GGTCGCCCCGTTAAAAGAGTA-3’) and CRT1-2 (CTGTTGCGA-TATAGATTAGGT-3’); MAG1-R1 (5’-GCGGTGATTTCCTGTTA-3’) and MAG1-R2 (5’-TGGCAGCCTCA AAAGTAT-3’); and RNR3-R1 (5’-GCTCTGGGCGCTTTT-3’) and RNR3-R2 (5’-CAGTGCGCCTTTT-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 OD600nm, 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 CRT10 (Figure 1). The predicted Crt10 contains leucine repeats at residues 253–267 (31) (Figure 1). WD-repeat proteins are found in 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 mec1Δ mutant

The crt10 mutant was originally isolated for its enhanced resistance to HU killing in a library screen. We compared the crt10Δ 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 crt10Δ::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 ultra-violet (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 mec1Δ inviability,
described. Plates were incubated for 3 days at 30°C before photographing. Strains used: (A) BY4741 (WT), WX1152 (crt10Δ), WX1153 (crt1Δ) and WX1154 (crt1Δ crt10Δ). (B) HK578-10A (WT), U952-3B (sml1Δ), WX1157 (crt1Δ), and WX1159 (sml1Δ crt10Δ). (C) The inviability of mec1Δ is partially rescued by deletion of CRT10. Tetrad results from a cross of WXY1158 (MEC1 SML1 crt10Δ) and U953-61A (mec1Δ sml1Δ CRT10) were dissected and the growth of each spore was followed by microscopic analysis. The representative picture was taken after 4 days incubation at 30°C.

whereas if it acts upon stalled replication fork, CRT10 deletion should not be able to rescue mec1Δ. The mec1Δ sml1Δ double mutant was crossed to crt10Δ in an isogenic background. Haploid spores recovered from 40 tetrads were genotyped by replica plating to appropriate media. No viable crt10Δ mec1Δ double mutant colonies were obtained. However, under the microscope, it was found that the mec1Δ mutant cells did not extend beyond two cell divisions, whereas the crt10Δ mec1Δ double mutant cells formed microcolonies containing up to several hundred cells (Figure 2C). This is in contrast to the sml1Δ mec1Δ double mutant from the same experiment, which formed visible colonies (data not shown). Hence, deletion of CRT10 appears to rescue mec1Δ cells from immediate death.

CRT10 belongs to the CRT1 regulatory pathway

Since deletion of CRT10 results in HU resistance and partially rescues the mec1Δ 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 crt10Δ crt1Δ and crt10Δ sml1Δ double mutants and comparing them to the corresponding single mutants with respect to HU resistance. The crt10Δ crt1Δ double mutant showed the same level of resistance to HU as the crt1Δ single mutant (Figure 2A), indicating that CRT10 belongs to the same pathway as CRT1. In contrast, the phenotypic effect of crt10Δ appears to be additive with sml1Δ (Figure 2B), suggesting that CRT10 does not belong to the same regulatory pathway as SML1.

The transcript level of RNR is elevated in crt10Δ mutants

Crt1 is an X-box DNA binding protein and represses the transcription of RNR2, RNR3, and RNR4 through recruitment of the corepressor complex Tup1-5sn6; deletion of CRT1 elevated the basal level expression of RNR3 25-fold (17). The above epistasis 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 crt10Δ mutants with or without MMS or HU treatment. Indeed, the RNR3-lacZ and RNR2-lacZ levels were elevated about 2-fold in crt10Δ 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, crt1Δ, crt10Δ single and the crt1Δ crt10Δ double mutants by β-gal assay. As shown in Table 3, the basal level of RNR3-lacZ was moderately elevated in the crt10Δ mutant and dramatically elevated in the crt1Δ mutant. Nevertheless, deletion of CRT10 does not further enhance RNR3-lacZ expression in the crt1Δ 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 crt10Δ dun1Δ 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
in HU resistance, cells carrying both deletions display a phenotype indistinguishable from that of dun1Δ mutant. Similarly, dun1Δ is epistatic to crt10Δ with respect to RNR3 expression, as deletion of CRT10 did not alter the reduced RNR3 induction in the dun1Δ mutant (Figure 4B).

Table 2. Relative steady-state transcript level

| Strain          | Relative transcript level* | Columns 1-6
|-----------------|-----------------------------|-------------
| Treatment       | HKS78-10D (WT) -HU            | +0.2 M HU   | WXY1158 (crt10Δ) -HU | +0.2 M HU |
|-----------------|-----------------------------|-------------|
| RNR3            | 1                           | 9.14        | 2.71              | 26.74     |
| MAG1            | 1                           | 2.32        | 1.27              | 2.19      |
| TUP1            | 1                           | 0.99        | 0.94              | 1.09      |
| SSN6            | 1                           | 0.26        | 0.38              | 0.50      |
| CRT1            | 1                           | 0.26        | 0.38              | 0.50      |

*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 crt1Δ and crt10Δ mutants

| Strain          | β-Galactosidase activity (Miller units) |
|-----------------|-----------------------------------------|
|                 | −MMS                                   | +0.02% MMS  |
| BY4741          | 1.6 ± 0.12                              | 48.9 ± 1.75 |
| WXY1153 (crt1Δ) | 72.5 ± 2.72                             | 101.3 ± 3.51|
| WXY1152 (crt10Δ)| 4.2 ± 0.32                              | 85.5 ± 1.60 |
| WXY1154 (crt1Δ-crt10Δ)| 73.8 ± 3.51           | 100.8 ± 2.74|

*All strains were transformed with pZZ2 (RNR3-lacZ).

These observations indicate that the HU resistance and increased RNR expression caused by CRT10 deletion require functional Dun1.

Cr10 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.

**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 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 (crt10Δ), WXY1155 (dun1Δ), WXY1156 (crt10Δ dun1Δ). (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.

**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 (crt1Δ) and WXY1155 (dun1Δ) 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 mecl null mutant, reminiscent of other suppressors of mecl 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.

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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