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Ccr4-Not Complex mRNA Deadenylase Activity Contributes to DNA Damage Responses in *Saccharomyces cerevisiae*

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ABSTRACT

DNA damage checkpoints regulate gene expression at the transcriptional and post-transcriptional level. Some components of the yeast Ccr4-Not complex, which regulates transcription as well as transcript turnover, have previously been linked to DNA damage responses, but it is unclear if this involves transcriptional or post-transcriptional functions. Here we show that *CCR4* and *CAF1*, which together encode the major cytoplasmic mRNA deadenylase complex, have complex genetic interactions with the checkpoint genes *DUN1*, *MRC1*, *RAD9*, and *RAD17* in response to DNA-damaging agents hydroxyurea (HU) and methylmethane sulfonate (MMS). The exonuclease-inactivating *ccr4-1* point mutation mimics *ccr4*Δ phenotypes, including synthetic HU hypersensitivity with *dun1*Δ, demonstrating that Ccr4-Not mRNA deadenylase activity is required for DNA damage responses. However, *ccr4*Δ and *caf1*Δ DNA damage phenotypes and genetic interactions with checkpoint genes are not identical, and deletions of some Not components that are believed to predominantly function at the transcriptional level rather than mRNA turnover, *e.g.*, *not5*Δ, also lead to increased DNA damage sensitivity and synthetic HU hypersensitivity with *dun1*Δ. Taken together, our data thus suggest that both transcriptional and post-transcriptional functions of the Ccr4-Not complex contribute to the DNA damage response affecting gene expression in a complex manner.

DNA damage checkpoints are signal transduction cascades activated by damage to the genome or replication stalling (reviewed in Nyberg *et al.* 2002; Longhese *et al.* 2003). After the initial recognition of damage to DNA or replication blocks, a series of phosphorylation events by checkpoint kinases enables the cell to mount an efficient response that includes a number of effects: arrest of the cell cycle until damage is repaired, regulation of the repair process, transcriptional activation of DNA damage-inducible genes, and in higher organisms, induction of apoptosis. The cell cycle resumes by a regulated process known as recovery when the damage is repaired (Vaze *et al.* 2002; Leroy *et al.* 2003).

In budding yeast, Mec1 and its subunit Ddc2 are the most upstream checkpoint kinases and may be able to sense some damage directly (Kondo *et al.* 2001; Melo *et al.* 2001; Rouse and Jackson 2002; Zou and Elledge 2003). Mec1 is required for three alternate DNA damage-signaling pathways that are characterized by the mediator proteins Mrc1, Rad9, and Rad17. While the Mrc1 pathway is believed to be specific for damage associated with DNA replication, the other two pathways are activated in response to a variety of lesions throughout the cell cycle (reviewed in Nyberg *et al.* 2002; Longhese *et al.* 2003). The mediators link Mec1 to the downstream effector kinases Rad53 and Chk1, thus enabling their activation (Sanchez *et al.* 1999; Alcasabas *et al.* 2001; Schwartz *et al.* 2002; Osborn and Elledge 2003). Together with Mec1, these kinases phosphorylate the checkpoint targets that execute the DNA damage response.

In addition to Rad53 and Chk1, *Saccharomyces cerevisiae* has a third effector kinase, Dun1, that acts mostly downstream of Rad53 in the checkpoint cascade. Dun1 has important roles in cell cycle arrest at the G_2/M phase (Gardner *et al.* 1999), transcriptional induction of damage-inducible genes [such as those coding for ribonucleotide reductase (RNR) subunits; Zhou and Elledge 1993; Gasch *et al.* 2001], phosphorylation and inhibition of the RNR inhibitor Sml1 (Zhao and Rothstein 2002), and regulation of repair pathways (Bashkirov *et al.* 2000). However, *dun1*Δ cells have higher genome instability rates than *rad53* mutants (Myung *et al.* 2001), indicating Rad53-independent functions of Dun1. Similar to Rad53 and its mammalian homolog CHK2, Dun1 contains an N-terminal forkhead-associated (FHA) domain, a phosphothreonine-binding module present in a large number of proteins (reviewed in Durocher

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and Jackson 2002; Hammet *et al.* 2003). We recently reported that the FHA domain of Dun1 interacts with the Pan2-Pan3 poly(A) nuclease (Hammet *et al.* 2002), a complex required for mRNA poly(A) tail length control (Brown and Sachs 1998). Dun1 and Pan2-Pan3 act together in regulating mRNA levels of the DNA repair gene *RAD5*, and *dun1*Δ *pan2*Δ and *dun1*Δ *pan3*Δ mutants are synthetically lethal in the presence of replication blocks (Hammet *et al.* 2002).

Shortening of the poly(A) tail by 3′→5′ exonucleases is the first step in mRNA turnover (reviewed in Parker and Song 2004). In addition to Pan2-Pan3, yeast contains another mRNA deadenylase, the Ccr4-Not complex, which is the major deadenylase responsible for initiating mRNA degradation (Daugeron *et al.* 2001; Tucker *et al.* 2001). The Ccr4-Not complex is conserved throughout evolution and in yeast consists of nine defined subunits: Ccr4, Caf1, Not1–Not5, Caf40, and Caf130 (reviewed in Collart 2003; Denis and Chen 2003). Ccr4 is the deadenylase catalytic subunit and contains a 3′→5′ exonuclease domain at its C terminus (Chen *et al.* 2002; Tucker *et al.* 2002). Single amino acid substitutions of catalytic residues in the Ccr4 exonuclease domain abolish its mRNA deadenylase activity *in vivo* and mimic most *ccr4*Δ phenotypes, indicating that its major cellular function is in mRNA degradation (Chen *et al.* 2002; Tucker *et al.* 2002). Although Caf1 also contains a nuclease domain and shows nuclease activity *in vitro* (Daugeron *et al.* 2001; Thore *et al.* 2003), *in vivo* studies do not support the notion that it is the active nuclease within the Ccr4-Not complex (Viswanathan *et al.* 2004). Nevertheless, Caf1 is absolutely required for exonuclease activity of Ccr4 *in vivo*, as *caf1*Δ mutants show the same deadenylation defects as *ccr4*Δ (Tucker *et al.* 2001).

Besides its role in regulating mRNA stability, Ccr4-Not also functions in the initiation and elongation phases of RNA polymerase II-dependent transcription (reviewed in Collart 2003; Denis and Chen 2003). Binding and phenotypic analyses indicate that the complex can be physically and functionally divided into two parts, Ccr4-Caf1 and the Not proteins (Bai *et al.* 1999; Maillet *et al.* 2000). Not1 is the core component of the complex and binds Ccr4 and Caf1 via its N terminus, while the other Not subunits are bound via the Not1 C terminus (Bai *et al.* 1999; Maillet *et al.* 2000). Although both functional parts of the Ccr4-Not complex contribute to transcriptional as well as post-transcriptional functions, Ccr4-Caf1 seems to be predominantly involved in mRNA deadenylation, whereas the Not proteins are believed to be primarily involved in transcription (Badarinarayana *et al.* 2000; Denis *et al.* 2001; Tucker *et al.* 2002; reviewed in Collart 2003).

Our finding that the poly(A) nuclease Pan2-Pan3 interacts with the checkpoint kinase Dun1 and has a critical role in survival of replication blocks indicated that post-transcriptional mechanisms of regulation of gene expression could be targeted by the DNA damage response pathway in yeast, and we have speculated that this could also involve the Ccr4-Not complex (Hammet *et al.* 2002). An involvement of the Ccr4-Not in responding to DNA damage is supported by the fact that mutants in several of its subunits, including the mRNA deadenylase catalytic subunit Ccr4, have been found to be sensitive to DNA-damaging agents in a number of whole-genome screens (Bennett *et al.* 2001; Hanway *et al.* 2002; Parsons *et al.* 2004; Westmoreland *et al.* 2004). To extend these analyses, here we have further investigated the role of the Ccr4-Not complex and its mRNA deadenylase activity in cellular DNA damage responses.

MATERIALS AND METHODS

Yeast strains: All strains are isogenic to Y136 (KY803) and are listed in Table 1. The deletion mutants were constructed using standard gene replacement methods. The E556A mutation was introduced in the genomic *CCR4* locus by PCR-based allele replacement (Erdeniz *et al.* 1997) to give the *ccr4-1* mutant.

DNA damage assays: To test the sensitivity to DNA-damaging agents, yeast strains were grown in YPD (yeast extract-peptone-dextrose) to logarithmic phase. Two microliters of 10-fold serial dilutions starting from A_{600} = 0.5 were spotted onto YPD plates or plates that contained HU or MMS at the doses indicated in the figures. The plates were photographed after 3–5 days.

For survival experiments over a 24-hr time course, overnight cultures were diluted to A_{600} = 0.2–0.3 and grown for 3 hr before HU was added to 0.1 m. Control samples were taken immediately before HU addition. Survival was followed over a period of 24 hr after addition of HU. Dilutions were plated on YPD plates and viable colonies were counted after 3–5 days depending on the growth rate of the strain. Survival was determined as a percentage of viable colonies relative to the control plate before HU addition. All incubations were done at 30°. For every strain, survival experiments were performed with at least two independent colonies.

Rad53 Western blots: To analyze Rad53 phosphorylation, cells were HU treated as above, washed three times in YPD, and released into YPD for up to 3 hr as indicated in the figure legend. Protein extracts were prepared using 8 m urea cracking buffer and processed for Rad53 Western blotting as described by Pike *et al.* (2001, 2003).

Northern blot analysis: For analysis of gene expression, cells were grown in YPD overnight and diluted to A_{600} = 0.2–0.3. After 3 hr of growth, cells were treated with 0.1 m HU for another 3 hr. RNA isolation and Northern analysis were performed as described (Hammet *et al.* 2000). Blots were probed with ^{32}P-labeled *RNR3* or *ACT1* probes (Pike *et al.* 2003). After exposure on PhosphorImager screens, the signals were quantified using Molecular Dynamics software. *RNR3* levels were normalized to levels of *ACT1*, and levels of *RNR3* message in HU-treated and untreated cells were calculated by comparing expression levels to basal levels of *RNR3* expression in the wild type.

RESULTS

***CCR4* and *CAF1* are required for DNA damage tolerance and show genetic interactions with *DUN1*:** To ana-

TABLE 1

Yeast strains used in this study

Strain	Genotype	Reference
Y136 (KY803)	*MAT***a** *trp1Δ1 ura3-52 gcn4 leu2::PET56*	Denis *et al.* (2001)
Y210	As Y136 but *not1-2*	Denis *et al.* (2001)
Y214	As Y136 but *not4::URA3*	Denis *et al.* (2001)
Y242	As Y136 but *dun1::LEU2*	This study
Y246	As Y136 but *dun1::LEU2 sml1::TRP1*	This study
Y294	As Y136 but *ccr4::klURA*	This study
Y297	As Y136 but *caf1::klURA*	This study
Y298	As Y136 but *not2::klURA*	This study
Y299	As Y136 but *not5::klURA*	This study
Y302	As Y136 but *not5::klURA dun1::LEU2*	This study
Y304	As Y136 but *ccr4::klURA dun1::LEU2 sml1::KAN*	This study
Y305	As Y136 but *caf1::klURA dun1::LEU2 sml1::KAN*	This study
Y310	As Y136 but *not3::klURA*	This study
Y317	As Y136 but *ccr4::klURA his3::TRP1*	This study
Y319	As Y136 but *caf1::klURA dun1::LEU2*	This study
Y332	As Y136 but *ccr4::klURA dun1::LEU2*	This study
Y333	As Y136 but *ccr4::klURA rad9::KAN*	This study
Y334	As Y136 but *ccr4::klURA rad17::KAN*	This study
Y335	As Y136 but *ccr4::klURA mrc1::KAN*	This study
Y336	As Y136 but *ccr4::klURA sml1::KAN*	This study
Y337	As Y136 but *caf1::klURA rad9::KAN*	This study
Y338	As Y136 but *caf1::klURA rad17::KAN*	This study
Y339	As Y136 but *caf1::klURA mrc1::KAN*	This study
Y340	As Y136 but *caf1::klURA sml1::KAN*	This study
Y345	As Y136 but *sml1::KAN*	This study
Y346	As Y136 but *rad9::KAN*	This study
Y347	As Y136 but *rad17::KAN*	This study
Y348	As Y136 but *mrc1::KAN*	This study
Y369 (ccr4-1)	As Y136 but *ccr4-E556A*	This study
Y370	As Y136 but *ccr4-E556A dun1::LEU2*	This study
Y379	As Y136 but *rpb9::KAN*	This study
Y380	As Y136 but *rpb9::KAN dun1::LEU2*	This study

kl, *Kluyveromyces lactis*.

lyze whether Ccr4-Caf1 deadenylase activity plays a role in the DNA damage response similar to Pan2-Pan3, *ccr4*Δ and *dun1*Δ *ccr4*Δ strains were tested for their ability to grow on plates containing HU or MMS. As shown in Figure 1A, *ccr4*Δ mutants were sensitive to both agents. Similar to what we observed for Dun1 and Pan2-Pan3, the *dun1*Δ *ccr4*Δ double mutants showed a dramatic increase in sensitivity to HU compared to either single mutant, suggesting that *CCR4* and *DUN1* might act toward the same goal in the cellular response to replication blocks, but provide separate functions. In contrast, the *dun1*Δ *ccr4*Δ cells were not synthetically sensitive to MMS (Figure 1A), potentially placing *CCR4* and *DUN1* in the same genetic pathway that acts upon alkylating DNA damage.

Caf1 is physically close to Ccr4 in the Ccr4-Not complex and the respective mutants have many phenotypes in common, including mRNA deadenylation defects (Tucker *et al.* 2001; reviewed in Collart 2003). We therefore tested the ability of *caf1*Δ cells to respond to DNA damage. *CAF1*-deleted cells showed even higher sensitivity to HU than did *ccr4*Δ mutants and were also mildly sensitive to MMS (Figure 1A). Similar to *dun1*Δ *ccr4*Δ, *dun1*Δ *caf1*Δ mutants showed synthetic sensitivity on HU plates. However, unlike *dun1*Δ *ccr4*Δ, *dun1*Δ *caf1*Δ cells were also more sensitive to MMS than were the respective single mutants (Figure 1A).

Impaired colony formation on plates containing DNA-damaging agents could reflect growth defects or an inability of the mutants to survive upon damage to the genome. To distinguish between these possibilities, we assayed the *dun1*Δ, *ccr4*Δ, *dun1*Δ *ccr4*Δ, *caf1*Δ, and *dun1*Δ *caf1*Δ strains for their ability to survive exposure to HU over a 24-hr time course. As shown in Figure 1B, the wild type could proliferate even in the presence of 0.1 M HU. *dun1*Δ, *ccr4*Δ, and *caf1*Δ mutants did not multiply, but remained viable, as >65% of the cells were able to form colonies on YPD plates after being removed from HU. In contrast, for the *dun1*Δ *ccr4*Δ and *dun1*Δ *caf1*Δ double mutants only 1–5% of the cells were viable

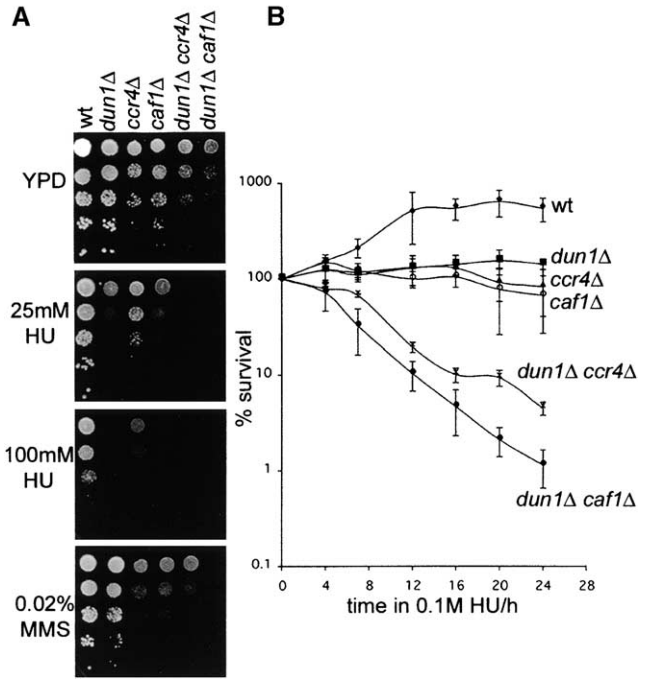

FIGURE 1.—DNA damage sensitivity of *ccr4*Δ and *caf1*Δ mutants and genetic interaction with *dun1*Δ. (A) Cells were grown to log phase and 10-fold serial dilutions starting from A_{600} = 0.5 were spotted on YPD plates with or without HU or MMS. (B) Time course survival assays of the indicated strains in 100 mM HU. Aliquots were taken immediately before and every 4 hr after addition of HU and plated on YPD plates. Viable colonies were counted after 3 days of growth and survival was determined on the basis of numbers of colonies before HU addition. Error bars represent the standard deviation. wt, wild type.

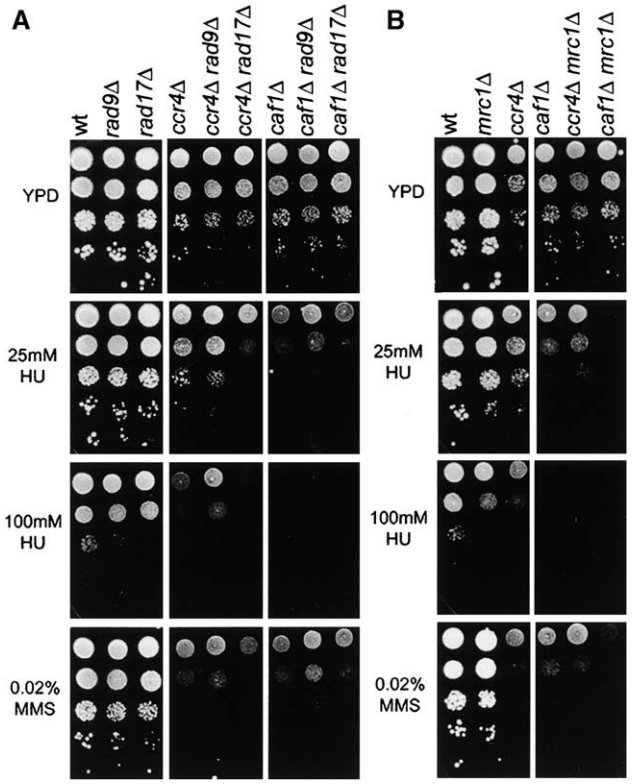

FIGURE 2.—Genetic interactions of *CCR4* and *CAF1* with *RAD9*, *RAD17*, and *MRC1*. Double deletion mutants of *rad9*Δ, *rad17*Δ (A), and *mrc1*Δ (B) with *ccr4*Δ or *caf1*Δ were assayed for their sensitivity to HU and MMS and compared to the respective single mutants. Cells were grown in YPD to log phase, diluted, and spotted onto YPD plates with or without 25 or 100 mM HU or 0.02% MMS and the plates were photographed after 3 days of growth at 30°. wt, wild type.

following 24 hr of exposure to HU. These data indicate that the observed synthetic sensitivity of the *dun1*Δ *ccr4*Δ and *dun1*Δ *caf1*Δ double mutants is due to HU-induced lethality. In other words, although Ccr4-Caf1 and Dun1 are required for cellular proliferation in the presence of replication blocks, only when Ccr4-Caf1 and Dun1-dependent functions are simultaneously deleted is exposure to HU actually lethal for the cells. Therefore, both Ccr4-Caf1 and Dun1 perform critical roles in survival after replication stress.

Complex genetic interactions of *CCR4* and *CAF1* with checkpoint mediators Rad9, Rad17, and Mrc1: To further explore the role of Ccr4 and Caf1 in the DNA damage checkpoint response we performed similar double-mutant analyses of *ccr4*Δ and *caf1*Δ mutants with deletions of the checkpoint genes *RAD9*, *RAD17*, or *MRC1*. As shown in Figure 2A, deletion of *RAD17* increased the sensitivity of *ccr4*Δ mutants on HU and also less severely on MMS, but had no effect on *caf1*Δ cells. In contrast, *rad9*Δ surprisingly led to moderately improved growth of *ccr4*Δ and *caf1*Δ strains on HU and, in the case of *caf1*Δ, also on MMS (Figure 2A). Deletion of *MRC1* in *ccr4*Δ or *caf1*Δ mutants mimicked the deletion of *DUN1*, in that both *ccr4*Δ *mrc1*Δ and *caf1*Δ *mrc1*Δ double mutants were more sensitive than the single mutants to HU, but only *caf1*Δ *mrc1*Δ also showed synthetic sensitivity to MMS (Figure 2B). The observed interactions with checkpoint proteins confirm that Ccr4 and Caf1 have a role in checkpoint pathways activated by DNA damage and replication blocks.

Ccr4 exonuclease activity is involved in the DNA damage response and genetic interaction with *DUN1*: To analyze the contribution of the deadenylase activity of Ccr4 to the DNA damage response, we introduced the E556A mutation into the genomic *CCR4* locus. Mutation of the E556 catalytic residue abrogates deadenylation activity of Ccr4 *in vivo* and *in vitro* (CHEN *et al.* 2002). The E556A change does not disrupt the stability of Ccr4 (CHEN *et al.* 2002) and, in agreement with that, the *ccr4 E556A* (*ccr4-1*) strain had better growth properties than *ccr4*Δ under basal conditions (Figure 3A control plate). However, with respect to DNA damage sensitivity, the exonuclease mutant behavior was similar to that in the deletion strain: it was sensitive to HU and MMS and in

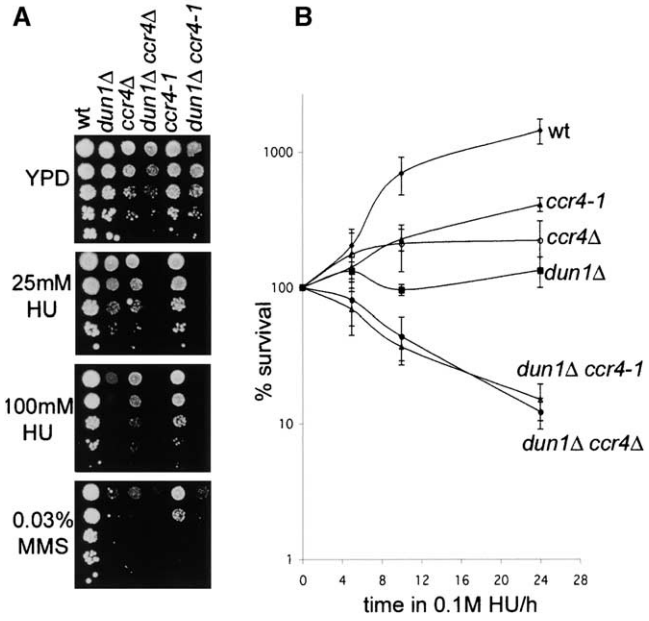

FIGURE 3.—Inactivation of the exonuclease activity of Ccr4 leads to DNA damage sensitivity. (A) Survival assays of *ccr4*Δ, *ccr4-1*, *ccr4*Δ *dun1*Δ, and *ccr4-1 dun1*Δ cells on HU and MMS plates as described for Figure 1A. (B) Time course survival assays in HU as in Figure 1B. wt, wild type.

combination with *dun1*Δ was synthetically sensitive to HU, but not to MMS (Figure 3A). We also performed quantitative survival assays in liquid 0.1 M HU cultures over a 24-hr time course (Figure 3B). In these assays, the *ccr4-1* catalytic domain mutant had slightly better proliferation properties than the *ccr4*Δ strain, but the HU survival rates of the *dun1*Δ *ccr4-1* double mutant were indistinguishable from those of *dun1*Δ *ccr4*Δ (Figure 3B), indicating that loss of mRNA exonuclease activity is responsible for the genetic interactions of *ccr4*Δ with *dun1*Δ. Altogether, the identical synthetic HU hypersensitivity of *dun1*Δ *ccr4-1* compared to *dun1*Δ *ccr4*Δ (Figure 3, A and B) and similar MMS hypersensitivity of *ccr4-1* compared to *ccr4*Δ (Figure 3A) demonstrate that Ccr4 mRNA deadenylase activity plays a critical role in response to DNA damage.

Contributions of transcriptional functions of the Ccr4-Not complex to the DNA damage response: Although Ccr4 is the catalytic subunit, Caf1 is believed to be equally important for mRNA deadenylase activity of the Ccr4-Not complex *in vivo* (TUCKER *et al.* 2001). Considering that the results with the *ccr4-1* catalytic domain mutant strongly support the involvement of Ccr4-Caf1 mRNA deadenylase functions in the DNA damage response, it was surprising that *ccr4*Δ and *caf1*Δ differed in some of their DNA damage-sensitivity phenotypes. For example, *caf1*Δ is more HU hypersensitive than *ccr4*Δ (Figures 1 and 2) and compared to *ccr4*Δ has different synthetic interactions in response to MMS with *dun1*Δ and in response to low-dose HU treatment with *mrc1*Δ (Figure 2B). A possible explanation to resolve this paradox was that nondeadenylase functions of the Ccr4-Not complex could also contribute to the DNA damage response. The main role of the Not proteins is believed to be in the regulation of transcription through modulation of the function of the general transcription factor TFIID (reviewed in COLLART 2003), and the respective mutants show only very subtle defects in mRNA deadenylation (TUCKER *et al.* 2002).

Therefore, to investigate if the transcriptional functions of the complex could also be involved in the DNA damage response, we analyzed the DNA damage sensitivity of *not1-2*, *not2*Δ, *not3*Δ, *not4*Δ and *not5*Δ mutants. In plate assays, *NOT2*, *NOT4*, and *NOT5* were required for normal cell growth in the presence of HU and, in the case of *NOT5*, also in the presence of MMS, while *not1-2* and *not3*Δ mutants had normal growth properties under these conditions (Figure 4A). In 24-hr HU survival experiments, *not2*Δ, *not4*Δ, and *not5*Δ strains showed a phenotype similar to that of *ccr4*Δ and *caf1*Δ (Figure 1B) in that they did not proliferate but remained largely viable (Figure 4B). Similar to *ccr4*Δ and *caf1*Δ (Figure 1B), *not5*Δ exhibited a dramatic synthetic lethality phenotype in concert with *dun1*Δ in response to HU (Figure 4B). Together with some discrepant DNA damage-sensitivity phenotypes between *ccr4*Δ and *caf1*Δ (Figures 1 and 2), these data support the notion that deadenylase-independent functions of the Ccr4-Not complex contribute to the DNA damage response, most likely involving its established role in the regulation of transcription.

The involvement of Ccr4-Not in multiple aspects of gene expression opens the possibility that disruption of the transcriptional process *per se* is responsible for the observed DNA damage-sensitivity phenotypes rather than specific functions of this complex. To address the specificity of our results with *ccr4-not* mutants, we deleted the gene encoding the nonessential RNA polymerase II subunit Rpb9 to analyze DNA damage phenotypes and the genetic interaction with *dun1*Δ (Figure 5). Rpb9 has been previously shown to be involved in transcription initiation and elongation and transcription-coupled DNA repair (TCR; HULL *et al.* 1995; HEMMING *et al.* 2000; LI and SMERDON 2002), and *rpb9*Δ mutants are hypersensitive to MMS and γ-irradiation (BENNETT *et al.* 2001; CHANG *et al.* 2002). As expected, *rpb9*Δ cells showed impaired growth properties on HU plates, but in contrast to *ccr4*Δ they did not exhibit a synthetic sensitivity phenotype with *dun1*Δ (in fact, *dun1*Δ partially suppressed the HU growth defect phenotype of *rpb9*Δ, suggesting that Dun1-dependent checkpoint functions may be involved in delaying cell cycle progression in the presence of DNA lesions that are normally repaired by TCR) (Figure 5A). Liquid survival assays indicated that the growth defect of *rpb9*Δ cells was fully reversible even after 24 hr in 100 mM HU, and again *rpb9*Δ and *dun1*Δ were not synthetic lethal under these conditions (Figure 5B). Therefore, these results indi-

cate that the genetic interactions between Ccr4-Not components and the Dun1 checkpoint kinase are specific for the functions of these proteins and not simply due to nonspecific effects of impaired transcription.

The HU sensitivity of *ccr4* and *caf1* mutants is independent of regulation of ribonucleotide reductase: HU causes replication blocks by inhibiting RNR, the enzyme required for biosynthesis of dNTPs. Therefore, one of the tasks of the replication checkpoint activated upon HU treatment is to increase the activity of RNR. This is achieved by Mec1-Rad53-Dun1 dependent transcriptional induction of genes coding for RNR subunits, such as *RNR3* (Zhou and Elledge 1993; Huang *et al.* 1998), phosphorylation and subsequent degradation of the RNR inhibitor Sml1 (Zhao *et al.* 2001; Zhao and Rothstein 2002) and by regulation of the subcellular localization of RNR subunits between the cytoplasm and the nucleus (Yao *et al.* 2003). Since the Ccr4-Not complex functions in gene expression, a plausible explanation for the observed sensitivity of *ccr4*Δ, *dun1*Δ *ccr4*Δ, *caf1*Δ, and *dun1*Δ *caf1*Δ mutants to HU was that they cannot induce *RNR* genes upon replication stress. We hence tested if *RNR3* was a target of Ccr4-Not. As expected *dun1*Δ cells were not able to significantly induce *RNR3* after treatment with HU (Figure 6, A and B). However, *ccr4*Δ and *caf1*Δ were proficient in transcribing *RNR3* upon replication stress, with *ccr4*Δ mutants showing even three to four fold higher levels of *RNR3* expression in HU than the wild type (Figure 6, A and B). Moreover, deletion of *CCR4*, but not *CAF1*, restored the ability of *dun1*Δ cells to produce *RNR3* mRNA upon replication stress at almost wild type levels (*dun1*Δ *ccr4*Δ +HU samples in Figure 6, A and B). Interestingly, although the *ccr4-1* exonuclease domain mutant had DNA damage phenotypes similar to the complete deletion, it did not lead to increased HU-induced *RNR3* expression by itself nor restoration of HU-induced *RNR3* expression in *dun1*Δ cells (Figure 6, A and B).

Deletion of *SML1* suppresses the phenotypes of checkpoint mutants that are associated with the inability to up-regulate RNR activity (Zhao *et al.* 1998). Therefore, another way of assessing whether lower RNR activity is the cause for the sensitivity of *ccr4*Δ, *dun1*Δ *ccr4*Δ, *caf1*Δ, and *dun1*Δ *caf1*Δ mutants to HU is to look at suppression effects of deleting *SML1*. As shown in Figure 6C, *sml1*Δ was able to partially suppress *ccr4*Δ and *caf1*Δ growth defects on HU plates, but it did not suppress the synthetic HU hypersensitivity of *dun1*Δ *ccr4*Δ and *dun1*Δ *caf1*Δ strains.

Collectively, these data indicate that the compromised activity of RNR upon treatment with HU is not the reason for the hypersensitivity of *ccr4*Δ, *dun1*Δ *ccr4*Δ, *caf1*Δ, and *dun1*Δ *caf1*Δ mutants to replication blocks.

Prolonged Rad53 activation in *dun1*Δ *ccr4*Δ mutants: To test if the severe sensitivity of *dun1*Δ *ccr4*Δ mutants to HU could be a consequence of impaired checkpoint

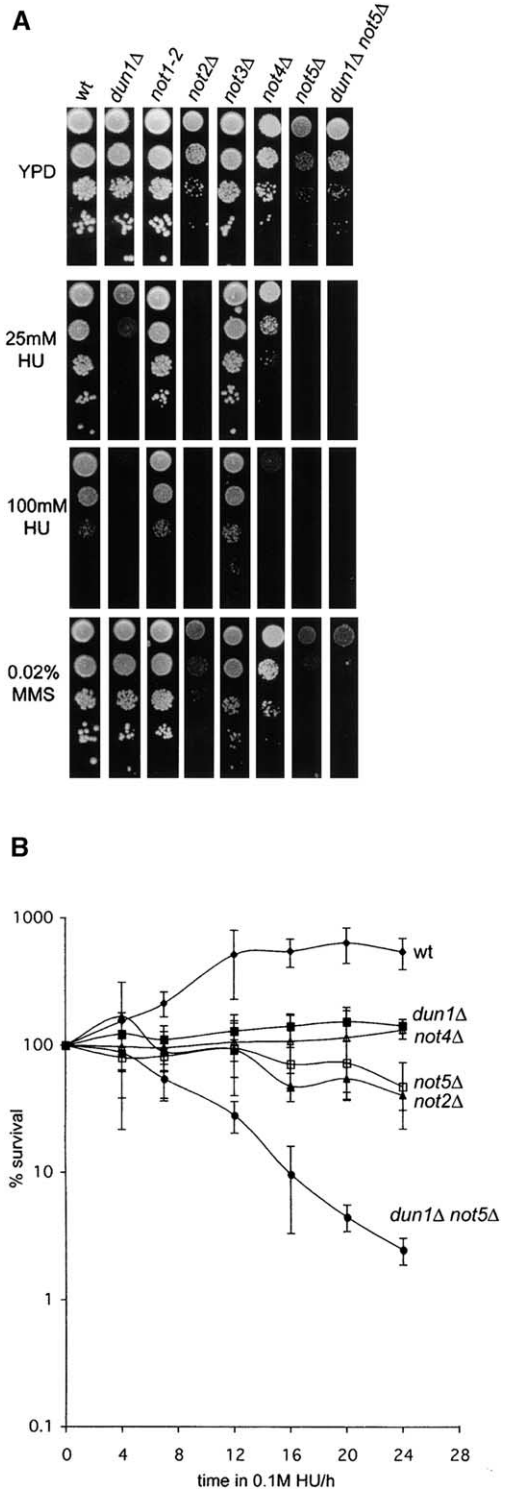

Figure 4.—Role of Not proteins in the DNA damage response. (A) Log phase cultures of wild-type or mutant strains were diluted and spotted on YPD plates with or without 25 or 100 mM HU or 0.02% MMS. *not2*Δ, *not5*Δ, and *dun1*Δ *not5*Δ strains were grown for 5 days before pictures were taken. All other strains were photographed after 3 days. (B) Survival after prolonged exposure to HU was measured as described for Figure 1B. wt, wild type.

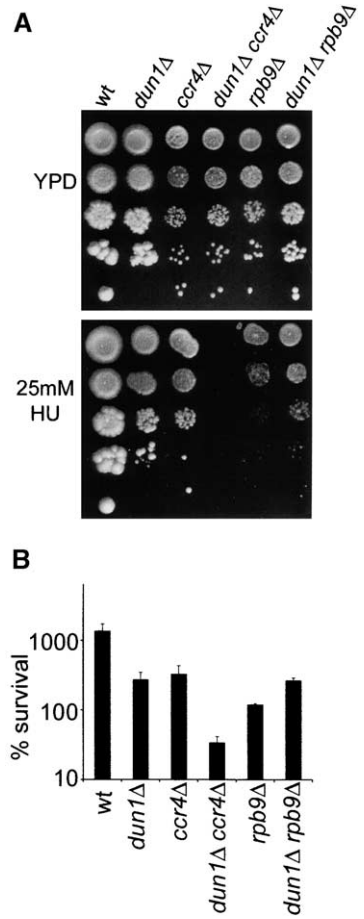

FIGURE 5.—Analysis of HU sensitivity of the *rpb9*Δ strain and its genetic interaction with *dun1*Δ. (A) Drop tests on HU-containing plates were done as in Figure 1A. Cells were photographed after 5 days of growth. (B) Survival after 24 hr in HU was determined as in Figure 1B. Error bars represent the standard deviation.

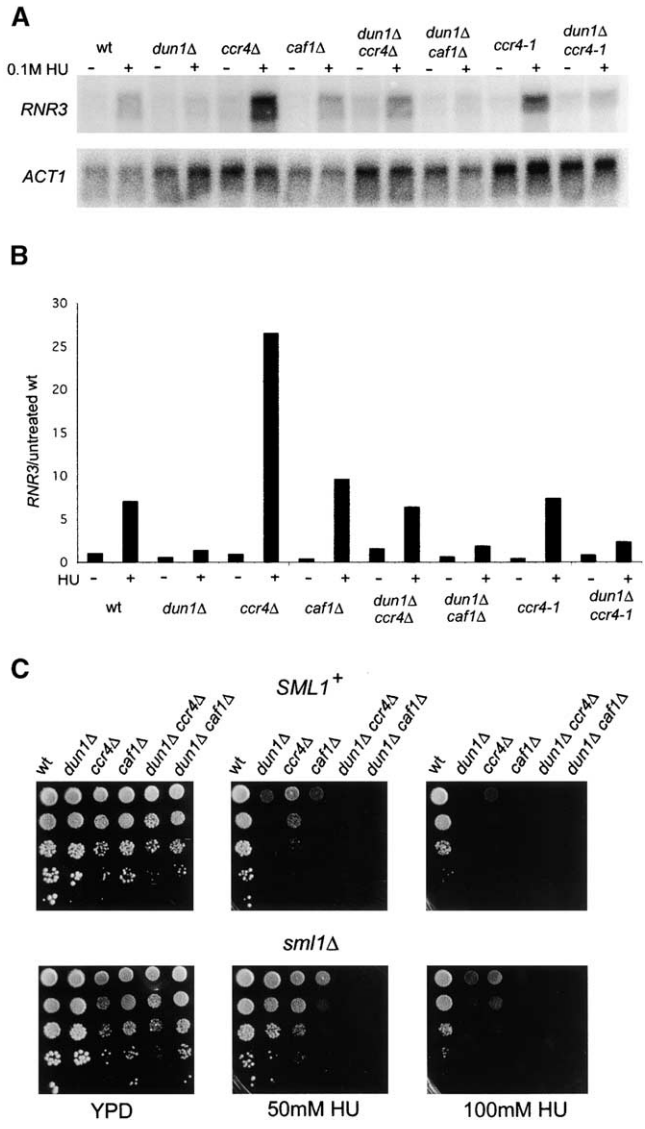

FIGURE 6.—Ccr4 and Caf1 are not required for expression of *RNR3* upon replication stress. (A) *RNR3* expression in the indicated strains was analyzed before and after treatment with 0.1 M HU for 3 hr. *ACT1* mRNA levels were used as loading control. (B) *RNR3* levels were normalized to *ACT1* levels and then expressed as fold difference compared to basal levels of expression in the wild type. (C) Analysis of the effect of *sml1*Δ on DNA damage sensitivity of *dun1*Δ, *ccr4*Δ, *dun1*Δ *ccr4*Δ, *caf1*Δ, and *dun1*Δ *caf1*Δ strains was done as described for Figure 1A. wt, wild type.

signaling, we analyzed Rad53 activation and inactivation kinetics in these strains. Rad53 is activated by phosphorylation that can be detected as slower mobility bands by Western blot analysis (PELLICIOLI *et al.* 1999), and in response to replication blocks the relative amount of shifted Rad53 correlates with the strength of the checkpoint signal (PIKE *et al.* 2004). In *dun1*Δ and *dun1*Δ *ccr4*Δ strains Rad53 was hyperphosphorylated compared to the wild type after 3 hr of 100 mM HU treatment, as well as after 16 hr HU treatment (Figure 7A). These data indicate that the increased HU lethality of *dun1*Δ *ccr4*Δ (Figure 1) is not the result of checkpoint failure. Interestingly, Rad53 activation was slightly reduced in *ccr4*Δ compared to the wild type (Figure 7A), presumably because higher *RNR3* levels in this strain (Figure 6, A and B) lead to reduced replicative damage in response to the same dose of HU.

Given that Rad53 was hyperphosphorylated in *dun1*Δ and *dun1*Δ *ccr4*Δ, it was possible that the decreased ability of the double mutant to form colonies after release from HU (Figure 1B) is the result of permanent cell cycle arrest due to irreversible checkpoint activation. To test this possibility, we monitored Rad53 inactivation in these strains after release from HU into normal medium (Figure 7B). In the wild type, the original Rad53 shift was approximately 50% reduced after 30 min and fully reversed after 1 hr. In contrast, Rad53 inactivation was delayed by 1 hr in the *dun1*Δ mutant and by another hour in the *dun1*Δ *ccr4*Δ double mutant (Figure 7B). These data indicate that *dun1*Δ *ccr4*Δ cells

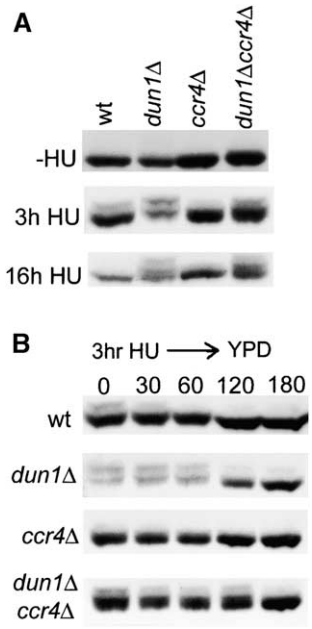

FIGURE 7.—Rad53 phosphorylation in *dun1*Δ, *ccr4*Δ, and *dun1*Δ *ccr4*Δ mutants. (A) Western blot analysis of Rad53 under basal conditions (−HU) and after 3 and 16 hr of 100 mM HU treatment in the indicated strains. (B) Western blot analysis of Rad53 before (0) and 30, 60, 120, and 180 min after release from 100 mM HU for 3 hr into HU-free YPD medium.

are significantly impaired in reversing checkpoint activation in the recovery from replication stress, but they are nevertheless able to fully turn off the checkpoint signal within 3 hr after HU release. As the 1-hr delay in Rad53 inactivation in *dun1*Δ *ccr4*Δ cells compared to *dun1*Δ (or 2 hr compared to wild type) would have only a minor effect on colony growth on HU-free plates after 3 days (Figure 1B), these results indicate that the increased HU-dependent lethality of the double mutant is not the result of a checkpoint recovery defect.

DISCUSSION

In this report we have shown that the exonuclease activity of the Ccr4-Caf1 mRNA deadenylase complex plays an important role in the cellular response to replicative DNA damage, in a manner that is synergistic with the Dun1 checkpoint kinase (Figures 1 and 3). Since the catalytic subunit Ccr4 and the noncatalytic subunit Caf1 are both essential for mRNA deadenylase activity of this complex (TUCKER *et al.* 2001), deletion of either subunit should have the same DNA damage-hypersensitivity effects. However, although *ccr4*Δ and *caf1*Δ mutants behaved overall in a similar manner, we found a number of important differences in their DNA damage-sensitivity profiles and synthetic genetic interactions with checkpoint genes (Figures 1 and 2). These discrepancies indicated that mRNA deadenylase-independent functions of the complex may also contribute to the DNA damage response. As Ccr4-Caf1 is part of the Ccr4-Not complex that functions in transcriptional regulation of gene expression in addition to mRNA deadenylation, we analyzed the role of other Not complex components in the DNA damage response and found that *not2*Δ, *not4*Δ, and *not5*Δ also resulted in replication block hypersensitivity and, in the case of *not5*Δ as an example, in a synthetic phenotype with *dun1*Δ similar to *ccr4*Δ or *caf1*Δ (Figure 4). Altogether, the most straightforward explanation for our findings is that both the transcriptional and the post-transcriptional functions of the Ccr4-Not complex play important functions in the DNA damage response.

A remarkable feature of *dun1*Δ *ccr4*Δ, *dun1*Δ *caf1*Δ, *dun1*Δ *not5*Δ double mutants, as well as double mutants of *dun1*Δ with a single residue substitution in an exonuclease catalytic residue (*ccr4-1*), was that they not only failed to grow in the continuous presence of the replication blocking agent HU, but also were unable to recover from replicative damage in survival assays in liquid cultures (Figures 1B, 3B, and 4B). Although *dun1*Δ *ccr4*Δ mutants were considerably delayed in reversing HU-induced Rad53 phosphorylation as a molecular marker for checkpoint activation, they were able to inactivate Rad53 within 3 hr of release from HU (Figure 7B). This indicates that *dun1*Δ *ccr4*Δ cells were able to process the checkpoint-activating DNA lesions into "neutral" products that were no longer recognized by the checkpoint machinery, yet were unable to sustain normal cell viability. As *dun1*Δ cells already have dramatically increased genome instability rates under basal conditions (MYUNG *et al.* 2001), a plausible explanation for this phenotype could be that *ccr4*Δ exacerbates this property of *dun1*Δ in response to HU by redirecting the repair of replicative DNA damage into inappropriate pathways with extensive genome rearrangements and loss of genetic material and subsequent loss of viability.

Considering its role in gene expression, there are two main possible mechanisms by which defects in the Ccr4-Not complex could affect DNA repair in "presensitized" *dun1*Δ mutants. First, changes to the transcriptional process *per se* could affect DNA repair, as damage is known to be more efficiently repaired by the TCR pathway in transcribed genes than in nontranscribed genes. However, in contrast to *ccr4*Δ, *caf1*Δ, and *not5*Δ, deletion of the nonessential RNA polymerase II subunit *RBP9*, which plays important roles in TCR and also gives rise to transcriptional defects, did not result in a synthetic HU hypersensitivity with *dun1*Δ. In addition, because the *ccr4-1* mutation most likely acts at the post-transcriptional level, a different mechanism seems more likely. This second mechanism could be that loss of some components of the Ccr4-Not complex in concert with *dun1*Δ leads to complex changes in cellular mRNA profiles that adversely affect the repair of replicative DNA lesions by shifting the equilibrium between opposing DNA re-

pair pathways. A similar mechanism has been invoked in the case of the related *dun1*Δ *pan2*Δ or *dun1*Δ *pan3*Δ phenotypes, where the increased replication block sensitivity could be attributed to increased *RAD5* expression (Hammet *et al.* 2002), but here we did not find significantly elevated *RAD5* mRNA levels in *dun1*Δ *ccr4*Δ strains (data not shown). Surprisingly, we found that HU-induced expression of *RNR3* was actually increased in *ccr4*Δ mutants (Figure 6), which should reduce the number of stalled replication forks; yet, despite presumably fewer lesions, this mutation resulted in HU hypersensitivity. With two candidate "culprits" ruled out, it will be interesting to see in more comprehensive array experiments how the expression of DNA repair genes is affected in mutants of the Ccr4-Not complex.

As already mentioned, *ccr4-not* mutants have been identified as sensitive to DNA damage induced by UV, ionizing radiation (IR), HU, MMS, and other DNA-damaging agents in several large-scale studies (Bennett *et al.* 2001; Hanway *et al.* 2002; Westmoreland *et al.* 2004). Some differences between these reports and our data are probably due to strain differences, use of diploid *vs.* haploid strains, and different DNA-damaging agents and conditions. Diploid *not3*Δ cells were reported to be IR hypersensitive (Westmoreland *et al.* 2004), whereas haploids are not hypersensitive to HU or MMS (Figure 5). Also, we found that although the mutants in the *NOT* genes were sensitive to UV, *ccr4*Δ and *caf1*Δ were not (data not shown). Westmoreland *et al.* (2004) also showed that diploid *ccr4*Δ cells have an extended *RAD9*-dependent cell cycle arrest after IR. The Rad9 pathway is not usually activated by replication blocks, but can be indirectly activated if primary replicative damage is processed into lesions that are sensed by the cell cycle-wide DNA damage pathways (Pike *et al.* 2004). Rad9 could therefore also contribute to Rad53 hyperphosphorylation and delayed recovery after HU release in *dun1*Δ and *dun1*Δ *ccr4*Δ cells (Figure 7), although deletion of *RAD9* improved growth of *ccr4*Δ and *caf1*Δ cells in the continuous presence of HU and MMS only very modestly (Figure 2A). Our results extend the findings of the large-scale screens by establishing that the mRNA deadenylase function of Ccr4-Not is involved in the DNA damage response and by comprehensively analyzing the genetic interactions of the complex with the alternate checkpoint pathways. In addition, our data suggest that the role of Ccr4-Not complex in DNA damage responses also involves mRNA deadenylase-independent functions, mostly likely related to its functions in the regulation of transcription. Not4, a subunit that we found was required for HU tolerance, is a potential ubiquitin ligase (Albert *et al.* 2002) and therefore this activity could be another means for Ccr4-Not to influence the DNA damage response, in a manner dependent or independent of its roles in transcription and mRNA turnover.

It is established that pathways activated by DNA damage target gene expression, and it is becoming more and more evident that, in addition to the well-characterized regulation of transcription (Zhou and Elledge 1993; Huang *et al.* 1998; Gasch *et al.* 2001), the DNA damage response also works by modulating post-transcriptional events in mRNA physiology. Emerging examples of post-transcriptional factors with links to the DNA damage response include the mammalian mRNA polyadenylation factor CstF (Kleiman and Manley 2001), the cytoplasmic *Schizosaccharomyces pombe* poly(A) polymerase Cid13 (Saitoh *et al.* 2002), the budding yeast poly(A) nuclease complex Pan2-Pan3 (Hammet *et al.* 2002), and now the Ccr4-Caf1 mRNA deadenylase complex. A question that is still unanswered is how post-transcriptional events in the cytoplasm are targeted by the DNA damage response, since the major components of checkpoint signaling pathways reside in the nucleus. Ccr4-Not could play a role in physically connecting these two processes via its dual role in both nuclear transcription and cytoplasmic mRNA turnover. The relation between these two functions of Ccr4-Not it is not yet clear, but it has been proposed that the interaction with the transcription machinery enables Ccr4-Not to associate cotranscriptionally with mRNA, before its export to the cytoplasm (Tucker *et al.* 2001). In such a way, the Ccr4-Not complex could enable the DNA damage signal to be transferred from the nucleus to the cytoplasm to act there in the post-transcriptional regulation of mRNA stability. This way, simultaneous targeting of nuclear transcriptional functions and cytoplasmic post-transcriptional functions of the Ccr4-Not complex by the checkpoint machinery could provide a means to facilitate a rapid switch in cellular mRNA profiles to adapt to the complex changes required for an efficient DNA damage response.

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