

DNA damage and replication stress induced transcription of *RNR* genes is dependent on the Ccr4–Not complex

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Received July 1, 2005; Revised October 5, 2005; Accepted October 16, 2005

ABSTRACT

Genetic experiments have indicated a role for the Ccr4–Not complex in the response to hydroxyurea (HU) induced replication stress and ionizing radiation in yeast. This response includes transcriptional induction of the four genes constituting the ribonucleotide reductase (RNR) enzymatic complex, *RNR1–4* and degradation of its inhibitor, Sml1p. The Ccr4–Not complex has originally been described as a negative regulator of RNA polymerase II (pol II) transcription, but it has also been implicated in mRNA turnover and protein ubiquitination. We investigated the mechanism of the HU sensitivity conferred by mutation of *CCR4–NOT* genes. We found that the ubiquitin protein ligase activity of Not4p does not play a role in HU induced Sml1p degradation. We show, however, that the HU sensitivity of *ccr4-not* mutant strains correlated very well with a defect in accumulation of *RNR2*, *RNR3* and *RNR4* mRNA after HU or methylmethane sulfonate (MMS) treatment. Chromatin immunoprecipitation (ChIP) experiments show that TBP, pol II and Set1p recruitment to the activated *RNR3* locus is defective in cells lacking *NOT4*. Moreover, *RNR3*-promoter activity is not induced by HU in these cells. Our experiments show that induction of *RNR* gene transcription is defective in *ccr4-not* mutant strains, providing an explanation for their sensitivity to HU.

INTRODUCTION

The Ccr4–Not complex is an essential evolutionarily conserved transcriptional regulator consisting of nine core subunits in yeast. It was originally described as a repressor of RNA polymerase II (pol II) mediated transcription (1–6).

Multiple functional and physical interactions between subunits of the Ccr4–Not complex and components of the general transcription machinery have been described. These components include subunits of TFIID (7–9), the mediator complex (2,10,11) and SAGA (2,12,13). Besides this, a role in positive regulation of transcription as well as in transcription elongation has been postulated (4,14). In addition to its roles in transcription, the Ccr4–Not complex subunits Ccr4p and Caf1p have been shown to represent the major cytoplasmic mRNA deadenylase in various species (15–19). Deletion of *NOT* genes, however, only mildly affects deadenylation (16). This is in agreement with the notion that the roles of Ccr4p and Caf1p do not completely overlap with those of the Not proteins (20). In several studies, it was noted that Not4p contains a Zn-finger motif (21,22), which we have identified as a RING-finger domain in its human ortholog CNOT4 (23). Proteins containing a RING-finger constitute a subgroup of ubiquitin protein ligases (E3s) (24). Indeed, CNOT4 displays RING-finger mediated E3 ligase activity *in vitro* (25). We found that the ubiquitin-conjugating enzyme (E2) UbcH5B was specifically required for this (26). Implication of the E3 activity in cellular processes and identification of CNOT4-substrates remain open issues.

Recently, it was shown that the Ccr4–Not complex plays a role in resistance to ionizing radiation and DNA damage or replication stress inducing chemicals (27,28). One of the major signaling pathways activated by DNA damage and replication stress contains the kinases Mec1p, Rad53p and Dun1p. Phosphorylation of Rad53p by Mec1p results in its activation, leading to subsequent phosphorylation of Dun1p (29,30). A key event for survival after DNA damage is an increase in dNTP levels in the cell (31), which is achieved by regulation of ribonucleotide reductase (RNR) activity (32). The RNR enzyme catalyses the transition of NDPs to dNDPs, which represents the rate limiting step in production of dNDPs needed for DNA replication and repair (32). In yeast, the subunits of the RNR complex are encoded by four genes, *RNR1–4* (33–36), which are transcriptionally induced

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following DNA damage and replication stress in a manner dependent on the Mec1p-Rad53p-Dun1p pathway (35,37,38). Notably, mutations in *RNR* genes (with the exception of *RNR3*) lead to sensitivity to DNA damage and hydroxyurea (HU), a chemical inhibitor of the RNR (36,39). In fact, many genes involved in DNA damage responses are sensitive to HU, reinforcing the link between DNA damage responses and dNTP levels (40,41). A genetic screen identified genes facilitating constitutive transcription of the *RNR* genes (42). For example, Crt1p, a repressor of *RNR* genes, binds to a specific DNA sequence in *RNR*-promoters (43). Dun1p-mediated hyperphosphorylation of Crt1p relieves its repressive function by reducing its DNA-binding capacity and enables derepression of the *RNR* genes (43). This involves both TFIID recruitment and SWI/SNF remodeling of the promoter region (44,45).

An additional mechanism to increase the enzymatic activity of the RNR complex depends on the depletion of Sml1p. The gene encoding Sml1p was identified as a suppressor of both *mec1* and *rad53* mutations, suggesting opposite roles for *SML1* and these checkpoint genes (46). Cells lacking *SML1* have higher basal levels of dNTPs and exhibit an increased resistance to DNA damaging agents (46). In addition, the Sml1 protein interacts with both the Rnr1p and Rnr3p subunits and inhibits RNR activity *in vitro* (47,48). In response to DNA damage, Sml1p is phosphorylated in a Mec1p-Rad53p-Dun1p dependent manner leading to its breakdown in the cell (49,50). Recent work has shown that the deadenylase activity of Ccr4p plays a role in tolerance to replication stress (28). Epistasis analysis showed an enhanced phenotype when *ccr4* or *caf1* mutations were combined with a deletion of *DUN1*, suggesting a mechanism distinct from derepression of *RNR* genes (28). No diminished *RNR3* mRNA expression was observed in *ccr4* and *caf1* mutant strains. In contrast, *ccr4Δ* cells showed a marked increase in *RNR3* mRNA accumulation, but this was not observed in a strain expressing a deadenylase deficient point-mutant of Ccr4 or in *caf1Δ* cells (28). *RNR3* expression in *NOT* deletion mutants was

not tested in this study. Moreover, it was postulated that the ubiquitin protein ligase potential of Not4 might play a role in the observed HU sensitivity phenotype (28).

Here, we describe a role for the Ccr4–Not complex in transcriptional induction of *RNR* genes in response to replication stress and DNA damage. In agreement with previous studies, we found that several subunits of the complex are important for tolerance to HU. Surprisingly, sensitivity to HU correlates very well with defects in accumulation of *RNR2*, *RNR3* and *RNR4* mRNA. We found that the Not4p subunit is required for efficient transcription induction of genes encoding the RNR complex by facilitating recruitment of various transcription factors, providing support for a positive role in transcription for the Ccr4–Not complex.

MATERIALS AND METHODS

Strains, genetic manipulation and plasmids

The *Saccharomyces cerevisiae* strains used in this study and their relevant genotypes are listed in Table 1. Cells were grown in YPD at 30°C. Yeast transformations were carried out using a LiAc method. ATG to STOP knock-out strains were made by one-step gene replacement. Genomic *not4L35A* mutants were obtained by integrating the pRS306-*not4L35A* into the *NOT4* locus in a *not4Δ* background using the *SmaI* restriction site in the *NOT4* promoter region (nt –226 relative to +1 ATG). Integrated mutants and gene disruptions were verified by PCR, phenotypic and/or western blot analysis. The *RNR3*-promoter-*GAL1* fusion plasmids were constructed by cloning PCR products into pRS316. The complete *GAL1* open reading frame (ORF) and its terminator region were fused to the *RNR3*-promoter using an introduced *BamHI* restriction site. *Pfu* polymerase was used to generate the DNA fragments.

HU sensitivity

Single colonies of the indicated strains were taken from plates, serially diluted (10-fold) and spotted on to YPD plates

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

Strain	Genotype	Source
BY4741	<i>mat a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	EUROSCARF
KMY106	Isogenic to BY4741 except <i>not2::KanMX</i>	This work
KMY58	Isogenic to BY4741 except <i>not4::KanMX</i>	EUROSCARF
KMY59	Isogenic to BY4741 except <i>not5::KanMX</i>	EUROSCARF
KMY107	Isogenic to BY4741 except <i>ccr4::KanMX</i>	EUROSCARF
KMY60	Isogenic to BY4741 except <i>caf1::KanMX</i>	EUROSCARF
KMY108	Isogenic to BY4741 except <i>caf4::KanMX</i>	EUROSCARF
KMY109	Isogenic to BY4741 except <i>caf16::KanMX</i>	EUROSCARF
KMY110	Isogenic to BY4741 except <i>caf120::KanMX</i>	EUROSCARF
KMY62	Isogenic to BY4741 except <i>caf130::KanMX</i>	EUROSCARF
MY1	<i>ura3-52, trp1-delta1, leu2::PET56 gal2 gcn4-delta1</i>	(1)
KMY102	Isogenic to MY1 except <i>not1-1</i>	Gift from M. Collart
KMY114	Isogenic to MY1 except <i>not1-2</i>	Gift from M. Collart
KMY103	Isogenic to MY1 except <i>not2::KanMX</i>	Gift from M. Collart
KMY104	Isogenic to MY1 except <i>not3::KanMX</i>	Gift from M. Collart
KMY97	Isogenic to MY1 except <i>not4::KanMX</i>	Gift from M. Collart
KMY105	Isogenic to MY1 except <i>not5::KanMX</i>	Gift from M. Collart
W303-1B	<i>mat α leu2-3112 his3-11 trp1-1 can1-100 ade2-1 ura3-1</i>	(62)
KMY2	Isogenic to W303-1B except <i>not4::KanMX</i>	(8)
KMY41	Isogenic to KMY2 except <i>not4L35A::URA3</i>	This work
KMY98	Isogenic to BY4741 except <i>sml1::URA3</i>	This work
KMY100	Isogenic to KMY58 except <i>sml1::URA3</i>	This work

containing various concentrations of HU. Cells were grown for 3–4 days at 30°C.

Western blotting

Cells were grown to $OD_{600} = 0.5$ –1 in YPD and subsequently treated with 200 mM HU. Samples (~2 OD U) were taken at the indicated time points. Extracts were prepared as described previously (51). Proteins were separated by 15% SDS–PAGE, transferred to a membrane and analyzed using Sm11 (47) and yTBP antiserum (52).

RNA extraction, northern blotting and cDNA synthesis

Total RNA was purified using the hot phenol extraction procedure. Briefly, 40 ml yeast cultures ($OD_{600} = 0.5$ –1) in YPD were added to 40 ml YPD containing 400 mM HU or 0.02% methyl-methane sulfonate (MMS) to obtain the final concentration of 200 mM HU or 0.01% MMS. Samples (7.5–10 ml) were taken and centrifuged for 2 min at 5000 r.p.m. (Sorvall Legend RT) Cell pellets were frozen on dry ice. Frozen cells were resuspended in 500 μ l of phenol:chloroform [5:1 (pH 4.7) at 65°C] and 500 μ l of TES buffer [10 mM Tris–HCl (pH 7.5), 1 mM EDTA and 0.5% SDS]. Cells were incubated for 1 h at 65°C and vortexed every 10 min for 20 s. The aqueous solution was extracted with phenol:chloroform [5:1 (pH 4.7) and RT) and with chloroform:isoamyl alcohol (25:1). Finally, RNA was collected by ethanol precipitation.

RNA (10 μ g) was separated by electrophoresis on 1% agarose gel containing 10 mM Na-phosphate (pH 6.7). RNA was then transferred to a nylon membrane and cross-linked by ultraviolet (UV) irradiation. The following PCR probes were radiolabeled using the RediPrime II kit (Amersham Pharmacia Biotech): *RNR1* (1–2667 nt, complete ORF), *RNR2* (1–1553 nt of genomic locus), *RNR3* (1–2610 nt, complete ORF), *RNR4* (1–1309 nt of genomic locus) and *ACT1* (324–1347 nt). Probes were hybridized overnight at 42°C. Blots were rinsed with 2 \times SSC at RT and sequentially washed with 2 \times SSC, 1 \times SSC, 0.5 \times SSC and 0.3 \times SSC (twice) for 15 min at 65°C. Membranes were subjected to autoradiography.

Total RNA (750 ng) was taken to prepare cDNA using random primers (Invitrogen) and the Superscript II kit (Invitrogen) according to the manufacturer's protocol.

Chromatin immunoprecipitation (ChIP)

Cells were grown in YPD to $OD_{600} \sim 0.6$ and subsequently treated with 200 mM HU by adding an equal volume of YPD containing 400 mM HU and incubation at 30°C for 2 h. Chromatin extracts were prepared essentially as described previously (53) with the exception that sonication was done using a bioruptor instrument (Diagenode). The settings were: medium strength, 7 \times 20/40 s on/off cycle in ice water. The average size of the sheared DNA was ~300–500 bp. Immunoprecipitations were performed with 12CA5 (anti-HA) or 8WG16 (anti-CTD of Rpb1) antibodies and performed as in (53). IgG–Sepharose fast flow beads (Amersham) were used to purify TAP–Set1–DNA complexes. Cross-links were reversed by incubation at 65°C overnight. DNA was purified over a Qiaquick PCR purification column (Qiagen) and analyzed by qPCR (see below).

Quantitative PCR analysis

A range of five 10-fold dilutions of the input material was used to quantify the ChIP efficiency by SYBR Green based quantitative PCR on a Chromo4 equipped PCR cycler (MJ Research). ChIP signals were normalized relative to the HMR (non transcribed mating type cassette on Chromosome III) signals.

Expression of *RNR3*, *GAL1* and *TUB1* mRNAs was analyzed by RT–PCR. Signals were quantified using a standard reference of yeast genomic DNA (Research Genetics) and normalized on *TUB1* expression. Primer sequences are available upon request.

RESULTS

Mutation of genes encoding for subunits of the Ccr4–Not complex confers sensitivity to HU

It was recently shown that components of the Ccr4–Not complex are required for efficient cell cycle progression after ionizing radiation and tolerance to HU (27,28). In agreement with this, we found that *not4* mutant cells display synthetic lethality with several genes implicated in DNA damage responses using synthetic genetic array analysis (K.W. Mulder, M.A. Collart and H.Th. Marc Timmers, manuscript in preparation). We confirmed this using various mutants of Ccr4–Not complex subunits by performing spot assays on plates containing increasing concentrations of HU using various genetic backgrounds to exclude strain specific observations. In accordance to published work (28), deletion of *NOT2*, *NOT4* and *NOT5* resulted in a pronounced HU sensitivity phenotype (Figure 1A and B and data not shown). A mutant of the essential *NOT1* gene [*not1-1* (*W1753R*), originally identified as *cdc39* (54)] also displayed HU sensitivity (Figure 1B). Traven and co-workers (28) recently reported that a different mutant of *NOT1* [*not1-2* (*S1298L,Q1316STOP*), (55)] is not sensitive to 100 mM HU. Interestingly, we

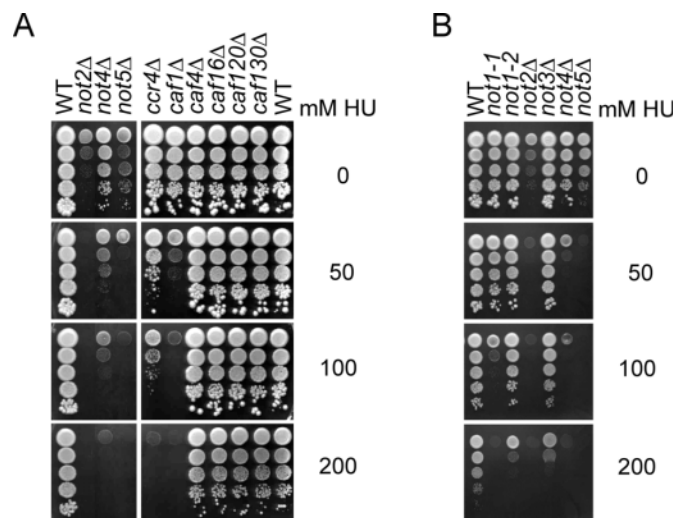


Figure 1. The Ccr4–Not complex is required for tolerance to HU. BY4741 (A) or MY1 (B) derived *ccr4-not* mutant strains were spotted on YPD plates containing the indicated concentrations of HU in 10-fold serial dilutions. Growth was assessed after 4 days.

found that this mutation, as well as deletion of *NOT3* resulted in sensitivity to 200 mM HU (Figure 1B). In addition, we observed that deletion of *CCR4* or *CAF1* also led to sensitivity to HU (Figure 1A). However, deletion of various Ccr4 associated factors (CAFs) that are described to be associated with the Ccr4–Not complex (6,11) did not show decreased viability in the presence of HU (Figure 1A and data not shown). Our results confirm and extend findings that the Ccr4–Not complex is involved in tolerance to HU.

Sml1p stability is not affected by the ubiquitin protein ligase potential of Not4p

Under normal growth conditions, Sml1p is associated with the RNR complex and inhibits its activity (47,48). In response to DNA damage or HU treatment, the Sml1 protein is phosphorylated by Dun1p and subsequently degraded (49,50). It has been postulated that the RING-finger dependent ubiquitin protein ligase (E3 ligase) activity of Not4 may be involved in the role of the Ccr4–Not complex in HU tolerance (28). We investigated this possibility by examining Sml1p levels after HU treatment. To avoid dissociation of the Ccr4–Not complex, we constructed a *not4* mutant encoding a defective RING-finger variant (Not4L35A), unable to interact with either Ubc4p or Ubc5p, and expressed it from its endogenous locus (our unpublished data). Interestingly, this mutant is sensitive to HU (K.W. Mulder, M.A. Collart and H.Th. Mare Timmers, manuscript in preparation). No significant effect on the reduction of cellular Sml1p levels after HU treatment could be observed in *not4L35A* cells, relative to WT cells (Figure 2A). Similar results were obtained using a *not4Δ* strain (data not shown). To further investigate this, we checked for synthetic interactions between *NOT4* and *SML1*. When HU sensitivity of *not4Δ* cells results from a defect in Sml1p depletion, it can be expected that deletion of *SML1* would suppress this phenotype. To test this, we constructed an *sml1Δ not4Δ* strain and tested its growth in the presence of HU. No significant rescue from HU sensitivity could be observed (Figure 2B), indicating that the HU sensitivity of *not4Δ* cells is independent of Sml1p regulation. Together, these results show that Not4p does not act as an E3 ligase to diminish Sml1 protein levels in response to HU treatment.

NOT4 is required for normal transcriptional induction of *RNR* genes following replication stress and DNA damage

A well-established reaction of the cell to DNA damage, or HU treatment, is transcriptional induction of the genes encoding RNR subunits (35,37,38). Since the effect of deletion of *NOT* genes on *RNR* gene expression was not previously determined, we examined whether the transcription regulatory function of Not4p is involved in this process. W303 *not4Δ* cells were grown to exponential phase and treated with HU, or MMS, for the indicated times. Unexpectedly, dramatic defects in *RNR3* and *RNR4* mRNA accumulation were observed after HU or MMS treatment in cells lacking *NOT4* (Figure 2C). In addition, small defects were observed in accumulation of *RNR1* and *RNR2* mRNAs (Figure 2C). These results indicate a role for Not4p in *RNR* transcription induced by DNA damage and replication stress.

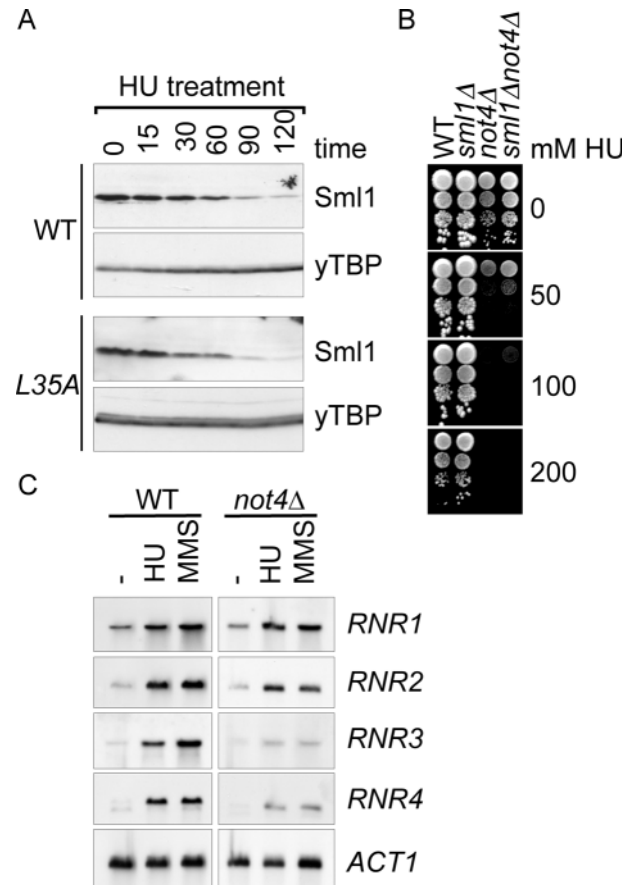


Figure 2. HU sensitivity of *not4Δ* cells is independent of Sml1p regulation but instead correlates with defective *RNR* gene transcription. (A) W303 WT and *not4L35A* cells were grown to mid-log phase and treated with 200 mM HU for the indicated time. Extracts were subjected to western blot analysis for Sml1p and yTBP protein levels. (B) BY4741 single and double mutants were serially diluted (10-fold) and spotted on YPD containing the indicated concentrations of HU. Growth was assessed after 4 days. (C) W303 WT and *not4Δ* cells were grown to mid-log phase and either non-treated or treated with 200 mM HU or 0.01% MMS for 2 h. Total RNA was extracted and subjected to northern blot hybridization using double stranded probes for the genes indicated on the right. Pictures are from the same blot and exposure.

Deregulated *RNR* gene induction is shared by several Ccr4–Not mutants

Next, we determined *RNR* mRNA induction following treatment with HU of other mutants of known Ccr4–Not complex subunits, in various genetic backgrounds (Figure 3). Northern blot analysis of *not1-1*, *not2Δ*, *not3Δ*, *not4Δ* and *not5Δ* strains in the MY1 background showed a strong correlation between HU sensitivity and defective *RNR* expression (Figure 3A). Accumulation of *RNR2*, *RNR3* and *RNR4* mRNAs are severely affected by deletion of *NOT2*, *NOT4* or *NOT5*. *RNR1* mRNA accumulation is only mildly affected in these strains and seems to be slightly delayed. Interestingly, the *not1-1* and *not3Δ* mutants display no defect in *RNR* gene transcription and show minimal (*not3Δ*) or moderate (*not1-1*) HU sensitivities (Figure 1B). Identical results were obtained for *not4Δ* and *not5Δ* cells using the BY4741 genetic background (Figure 3B). Given the fact that *ccr4Δ* and *caf1Δ* cells are also sensitive to HU (Figure 1A), we tested *RNR* transcription following HU treatment (Figure 3B). In contrast to what was

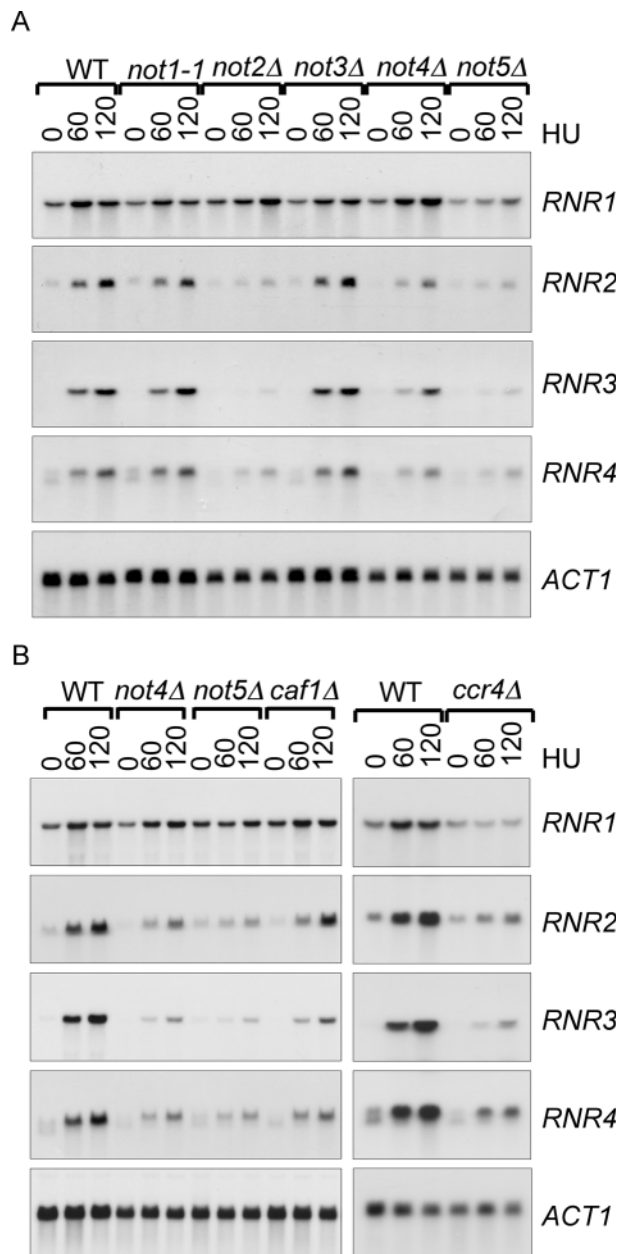


Figure 3. Ccr4-Not complex mutants display defects in accumulation of *RNR* mRNA. MY1 (A) or BY4741 (B) derived Ccr4-Not mutants were grown to mid-log phase and treated with 200 mM HU for the indicated time (min). RNA was subjected to northern blot analysis as in Figure 2C.

found previously (28), we observed severe defects in *RNR* mRNA accumulation in *caf1Δ* and *ccr4Δ* strains. This discrepancy might be due to differences in strain backgrounds. Since DNA damage and replication stress lead to activation of the same signal transduction pathway (35,37,38), we also determined *RNR* transcription in *not4Δ*, *caf1Δ* and *ccr4Δ* strains after MMS treatment. Although less pronounced than after HU treatment, *ccr4Δ* showed a defect in *RNR3* mRNA accumulation (diminished about 3-fold, Figure 4). Interestingly, in this experiment *RNR1* mRNA levels were elevated in the *ccr4Δ* and *caf1Δ* strains. Although not observed reproducibly (Figure 3B), this could indicate that Ccr4p and Caf1p are specifically involved in regulation of *RNR1* mRNA stability.

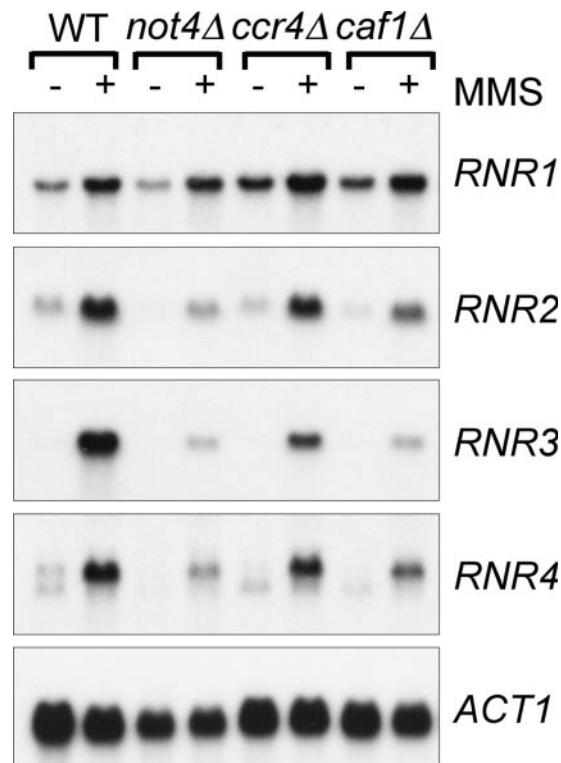


Figure 4. The Ccr4-Not complex is required for transcriptional induction of *RNR* genes after DNA damage induced by MMS. Cells were grown to mid-log and subsequently treated with 0.01% MMS for 2 h. RNA was extracted and subjected to northern blot analysis as in Figure 2C.

However, deletion of *NOT4* or *CAF1* led to strongly diminished levels of *RNR2*, *RNR3* and *RNR4* mRNA after MMS treatment and this is comparable to the reduced induction after HU treatment (Figure 3).

Together, these experiments show that expression of *RNR* genes following HU or MMS treatment in cells lacking Ccr4-Not complex components is severely affected. These results correlate very well with the observed sensitivity to HU (Figure 1).

Not4p is required for recruitment of TBP, pol II and Set1p to the activated *RNR3* locus

To investigate the defect in *RNR* mRNA expression further, we performed ChIP experiments to assess the recruitment of various transcription factors to the *RNR3* locus. Using antibodies recognizing the CTD of pol II, we determined its recruitment to the activated *RNR3* gene. As shown in Figure 5B, pol II was recruited to the locus in WT, but not in *not4Δ* cells following HU treatment. Remarkably, we observed elevated pol II-binding to the transcribed region in the *not4Δ* cells compared to WT, under non-inducing conditions. This does not result in higher basal *RNR3* mRNA levels (see Figures 3 and 4). Similar results were obtained using an antibody against the Rpb3 subunit of pol II (data not shown). Possibly, Not4p plays a role in preventing mislocalization of pol II, but this requires more extensive analysis. To confirm and extend these observations, we determined recruitment of Set1p to the *RNR3* locus. Previous work has shown that recruitment of the Set1 complex is dependent on pol II

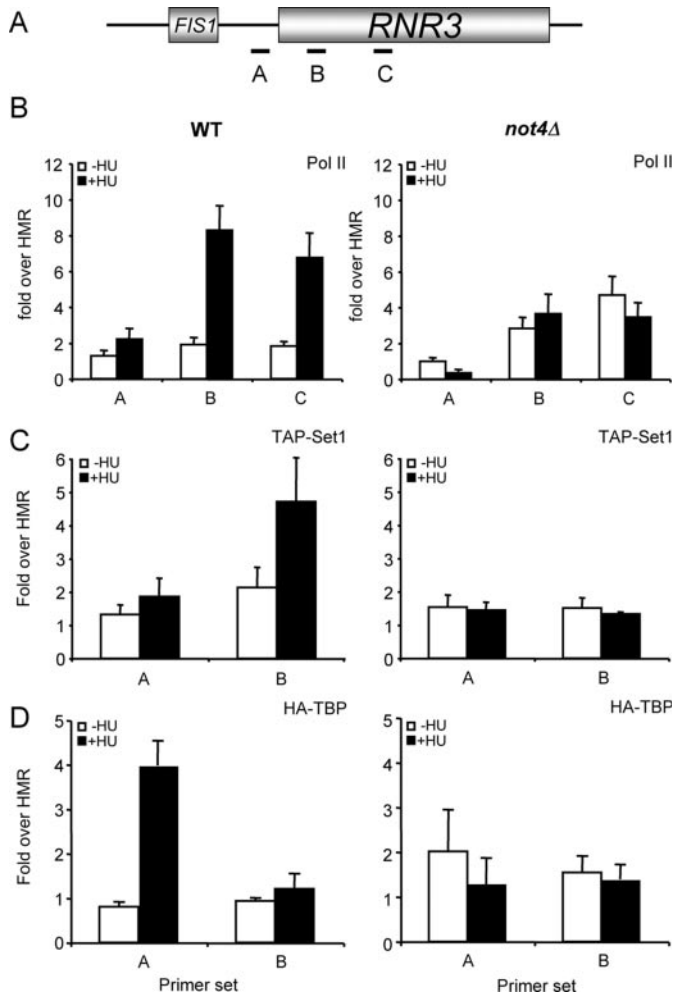


Figure 5. Not4 plays a critical role in recruitment of transcription factors to the *RNR3* locus after HU treatment. (A) Schematic representation of the *RNR3* locus, A–C represent the regions analyzed by qPCR after ChIP. pol II (B), TAP-Set1p (C) and HA-TBP (D) recruitment was determined in WT and *not4Δ* cells treated with HU for 2 h.

(56). As expected, Set1p was not recruited in *not4Δ* cells whereas WT cells showed a clear increase after HU treatment (Figure 5C). Although pol II is present on the 5' transcribed region of the *RNR3* gene under normal conditions in *not4Δ* cells, this was not reflected by elevated levels of Set1p (Figure 5C). Since pol II and Set1p were not recruited, we analyzed whether an upstream event, TBP recruitment, could still take place. As expected, TBP was specifically recruited to the region containing the TATA-box (region A) in WT cells (Figure 5D). In contrast, in cells lacking *NOT4* HU treatment does not result in recruitment of TBP to DNA (Figure 5D).

An *RNR3*-promoter driven reporter mimics endogenous gene regulation

To confirm that the observed effects in recruitment of transcription machinery components caused specific defects in *RNR3*-promoter activity after HU treatment, we constructed fusions between the *RNR3*-promoter region (either –600 or –850 bp from ATG) and the *GALI* ORF and terminator sequence. We determined that endogenous *GALI* promoter

activity and mRNA stability was not affected by deletion of *NOT4* (data not shown), indicating that differences arising from these reporter constructs would reflect *RNR3*-promoter activity. The 850 bp promoter region resembled a previously published reporter construct (42), whereas the 600 bp promoter region represents the *FIS1-RNR3* intergenic region. WT and *not4Δ* cells were transformed with the indicated reporter plasmids or an empty vector control. After treatment with HU, RNA was extracted and mRNA expression was analyzed by northern blot hybridization. *GALI* mRNA was readily detected after HU treatment in WT cells (Figure 6A), whereas *GALI* mRNA induction was reduced (~2.5-fold) in *not4Δ* cells, reflecting the defect in endogenous *RNR3* mRNA accumulation (~5-fold diminished) (Figure 6A). Although the 600 bp promoter construct seems to be more readily induced compared to the 850 bp construct, this difference is observed in both WT and *not4Δ* cells (Figure 6A). This indicates that the 600 bp *FIS1-RNR3* intergenic region is sufficient for HU induced activation and Ccr4–Not dependence. We also noted that *ACT1* mRNA levels are generally reduced in *not4Δ* cells (see also Figures 3 and 4). However, this is not due to differences in loading total RNA, since the 25S and 18S ribosomal RNA signals are equal between WT and *not4Δ* (Figure 6A). The northern blot results were confirmed using RT quantitative PCR. Total RNA of cells containing the 850 bp *RNR3*-promoter region was used to synthesize cDNA. Tubulin (*TUB1*) mRNA was used as an internal control. *RNR3* cDNA is detected in a time dependent manner in cells containing the *RNR3*-promoter construct, as well as in cells containing an empty plasmid (Figure 6B). WT versus *not4Δ* comparison shows a ~4.5-fold difference in *RNR3* expression (Figure 6B). This difference is even more pronounced when analyzing the *RNR3*-promoter driven *GALI* mRNA levels (~13-fold). As expected, no *GALI* mRNA signal was observed in the cells containing an empty vector (Figure 6B). Together with the ChIP data (Figure 5), the analysis of *RNR3*-promoter derived mRNA levels (Figures 3,4 and 6) show that *RNR3*-promoter activity is compromised in *not4Δ* cells. This indicates that the Ccr4–Not complex is involved in regulation of HU induced *RNR* gene transcription and provides an explanation for the observed HU sensitivity of *ccr4-not* mutant strains.

DISCUSSION

The Ccr4–Not complex was shown previously to be a repressor of transcription, possibly by blocking transcription initiation by preventing binding of TFIID to DNA (1,4,12,55,57–59). Here, we provide evidence that the Ccr4–Not complex is required for transcription activation of the *RNR3*-promoter by facilitating TBP, pol II and Set1p recruitment (Figure 5). This was confirmed using an *RNR3*-promoter driven reporter assay (Figure 6). The observed defects in induction of *RNR* genes strongly correlate with the sensitivity to HU displayed by various *CCR4-NOT* deletions (Figures 1 and 3), indicating the importance of the complex during replication stress and DNA damage.

Regulation of the RNR by the Ccr4–Not complex

Previous work has genetically linked the Ccr4–Not complex with a DNA damage response pathway (27,28). We confirmed

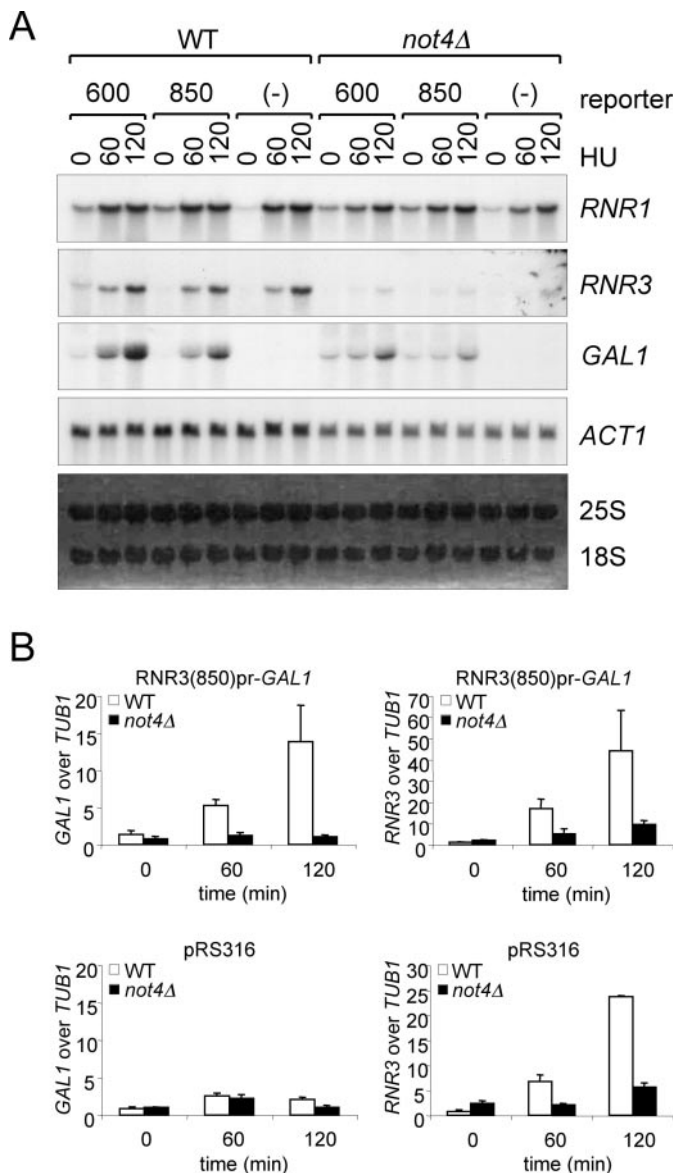


Figure 6. Not4 is required for transcription activation of the *RNR3*-promoter. (A) Plasmids containing a fusion of the *RNR3*-promoter (600 and 850 bp, respectively) and the *GAL1* ORF and terminator, or an empty vector (pRS316), were used to transform WT and *not4Δ* strains. Cells were grown to mid-log phase and treated with 200 mM HU for the indicated time (min). RNA was extracted and subjected to northern blot analysis as in Figure 2C. (B) cDNA was generated from RNA samples in A (850 bp *RNR3*-promoter construct) and subjected to quantitative PCR analysis using *RNR3* and *GAL1* specific primers. Values are depicted as fold over $t = 0$ after normalization to *TUB1* cDNA levels.

that several deletion mutants of Ccr4–Not complex subunits display a marked sensitivity to HU (Figure 1A and B). Well-documented responses to DNA damage include transcriptional induction of the four genes encoding the RNR complex, *RNR1–4*, and degradation of Sml1p (35–37,39). An indistinguishable response is elicited by treatment with HU, resulting in replication stress (35,36,39). The Sml1 protein can interact with both Rnr1p and Rnr3p, resulting in inhibition of the enzymatic activity of the RNR complex (46–48). Direct phosphorylation by Dun1p leads to a decrease in Sml1p levels in

the cell by an as yet-undefined mechanism (50). We considered the possibility that the ubiquitin protein ligase function of Not4 was directly involved in degradation of Sml1p following Dun1p-mediated phosphorylation. However, we found that expressing an ubiquitin ligase deficient variant of Not4p or deleting *NOT4* completely had no effect on HU induced Sml1p depletion (Figure 2A and data not shown). In agreement with the possibility that Sml1p is degraded independently of the ubiquitin proteasome pathway, we observed that HU induced Sml1p degradation was not affected by MG132, a 26S proteasome inhibitor (data not shown). However, increased levels of Sml1p were observed under normal conditions in the presence of MG132 (data not shown). In addition, deletion of *SML1* did not suppress the *not4Δ* HU sensitivity, indicating that regulation of Sml1p is not dependent on the Ccr4–Not complex (Figure 2B).

Previous work showed that mutations in *RNR1*, *RNR2* or *RNR4* result in sensitivity to DNA damage or HU treatment (35–37,39). In addition, mutations disabling a normal induction of *RNR* genes are sensitive to HU and DNA damaging agents (42). This suggests a causative link between deregulation of the *RNR* genes and sensitivity to HU treatment. Both HU sensitivity and defective *RNR* gene induction are observed in Ccr4–Not complex mutants, suggesting a direct relationship between the HU sensitivity and the observed defects in *RNR* gene expression in Ccr4–Not mutants.

Involvement of the Ccr4–Not complex in *RNR* gene transcription

The mechanism by which the Ccr4–Not complex is involved in tolerance to HU was unidentified previously. However, recent work by Traven and co-workers (28) showed involvement of the deadenylase activity of Ccr4p and Caf1p in resistance to HU in a pathway parallel to Dun1p, as derived from epistasis analysis. In addition, the authors showed that in *ccr4Δ* cells, *RNR3* mRNA accumulation after HU treatment was increased compared to WT. However, this was not the case in *ccr4-1* cells, expressing an exonuclease domain mutant (E556A) abrogating the deadenylation activity of Ccr4p (28). It seems difficult to reconcile the increased *RNR3* mRNA levels in *ccr4Δ* cells with sensitivity to HU, especially since this was not observed in the catalytically inactive *ccr4-1* mutant. Interestingly, this mutant was not sensitive to HU, but only displays a synthetic sensitivity when combined with a *DUN1* deletion (28). In contrast, we found that *ccr4Δ* and *caf1Δ* strains display reduced *RNR3* mRNA accumulation in response to HU treatment or DNA damage (Figures 3 and 4). At present, the reason for this discrepancy remains unclear. Our data suggest that, in addition to their documented roles in mRNA degradation, Ccr4p and Caf1p may have a positive function in transcription in the context of the Ccr4–Not complex.

A growing number of factors including repressors, chromatin remodelers and basal transcription factors, have been implicated in regulation of *RNR3* gene transcription (43–45, 60,61). The SWI/SNF chromatin-remodeling complex was shown to be required for remodeling and efficient induction of *RNR* genes after MMS or HU treatment (44,45). The activity of this complex on the *RNR3*-promoter is facilitated by the SAGA complex (45) and dependent on general transcription

factors including pol II and TFIID (44,45). At present it is not clear whether the effects we observe on induction of *RNR* genes are direct or indirect via deregulation of critical factors involved in this process. However, it seems unlikely that these factors would include the transcriptional repressor Crt1p, since we did not detect major effects on basal levels of *RNR* mRNAs. Our observation that TBP is not recruited in *not4Δ* cells, combined with the documented genetic and physical interactions between the Ccr4–Not complex and TFIID (2,7–9,12), could indicate a direct role for the Ccr4–Not complex in regulation of *RNR* genes after DNA damage and replication stress. Although these interactions have thus far been suggested to result in transcription repression, they might also contribute positively to transcription of specific genes. In addition, a direct interaction between Not2p and the SAGA subunit Ada2 was described (13) and could also be involved in regulation of *RNR* gene activation. Both these models would require the Ccr4–Not complex to be present on the promoter regions of *RNR* genes. Thus far, our efforts to crosslink Ccr4–Not complex components to the *RNR3* locus, under both normal and induced conditions, failed to provide evidence for this (data not shown). Further investigation is required to obtain more efficient protocols for cross-linking the Ccr4–Not complex to DNA. Regardless, our results clearly show that the Ccr4–Not complex contributes to the cellular DNA damage response by facilitating proper transcription induction of *RNR* genes.

Taken together, we showed that various subunits of the Ccr4–Not complex are involved in tolerance to HU induced replication stress and that this strongly correlates with a requirement for the Ccr4–Not complex in *RNR* gene expression after HU treatment or DNA damage. In addition, we found that Not4p facilitates TBP, pol II and Set1p recruitment to the *RNR3* locus after HU treatment, establishing a positive function for the Ccr4–Not complex in transcription induction of specific pol II transcribed genes. Our work provides an explanation for the role of this complex in DNA damage response pathways can serve as a framework for further analysis of positive functions in transcription for the Ccr4–Not complex.

ACKNOWLEDGEMENTS

We thank Dr M.A. Collart and Dr A.F. Stewart for yeast strains and Dr R. Rothstein and Dr P.A. Weil for providing Sml1 and yTBP antisera, respectively. We are grateful to Dr M.A. Collart and members of the Timmers laboratory for helpful discussions and technical advice and assistance. This work was supported by grants from The Netherlands Organization for Scientific Research (NWO-MW Pionier #900-98-142 and NWO-CW #700-50-034) and European Union (Improving Human Potential RTN2-2001-00026). Funding to pay the Open Access publication charges for this article was provided by NWO-CW #700-50-034.

Conflict of interest statement. None declared.

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