Ccr4 contributes to tolerance of replication stress through control of *CRT1* mRNA poly(A) tail length

Robert N. Woolstencroft[1,2], Traude H. Beilharz[3], Michael A. Cook[1,2], Thomas Preiss[3,4], Daniel Durocher[1,2,*] and Mike Tyers[1,2,*]

[1]Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, M5G 1X5, Canada
[2]Graduate Department of Molecular and Medical Genetics, University of Toronto, Toronto, M5S 1A8, Canada
[3]Victor Chang Cardiac Research Institute (VCCRI), Darlinghurst (Sydney), NSW 2010, Australia
[4]School of Biotechnology and Biomolecular Sciences and St Vincent's Clinical School, University of New South Wales, Sydney, NSW 2052, Australia

*Authors for correspondence (e-mail: durocher@mshri.on.ca; tyers@mshri.on.ca)

Accepted 21 August 2006
Journal of Cell Science 119, 5178-5192 Published by The Company of Biologists 2006
doi:10.1242/jcs.03221

Summary

In *Saccharomyces cerevisiae*, DNA replication stress activates the replication checkpoint, which slows S-phase progression, stabilizes slowed or stalled replication forks, and relieves inhibition of the ribonucleotide reductase (RNR) complex. To identify novel genes that promote cellular viability after replication stress, the *S. cerevisiae* non-essential haploid gene deletion set (4812 strains) was screened for sensitivity to the RNR inhibitor hydroxyurea (HU). Strains bearing deletions in either *CCR4* or *CAF1*/*POP2*, which encode components of the cytoplasmic mRNA deadenylase complex, were particularly sensitive to HU. We found that Ccr4 cooperated with the Dun1 branch of the replication checkpoint, such that *ccr4*Δ *dun1*Δ strains exhibited irreversible hypersensitivity to HU and persistent activation of Rad53. Moreover, because *ccr4*Δ and *chk1*Δ exhibited epistasis in several genetic contexts, we infer that Ccr4 and Chk1 act in the same pathway to overcome replication stress. A counterscreen for suppressors of *ccr4*Δ HU sensitivity uncovered mutations in *CRT1*, which encodes the transcriptional repressor of the DNA-damage-induced gene regulon. Whereas Dun1 is known to inhibit Crt1 repressor activity, we found that Ccr4 regulates *CRT1* mRNA poly(A) tail length and may subtly influence Crt1 protein abundance. Simultaneous overexpression of *RNR2*, *RNR3* and *RNR4* partially rescued the HU hypersensitivity of a *ccr4*Δ *dun1*Δ strain, consistent with the notion that the *RNR* genes are key targets of Crt1. These results implicate the coordinated regulation of Crt1 via Ccr4 and Dun1 as a crucial nodal point in the response to DNA replication stress.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/119/24/5178/DC1

Key words: Ccr4 mRNA deadenylase, Chk1, Crt1, Dun1, poly(A) tail, Replication checkpoint, Transcription

Introduction

Replicating cells treated with the ribonucleotide reductase (RNR) inhibitor hydroxyurea (HU) pause or slow down replication fork progression (Branzei and Foiani, 2005). In budding yeast, replication fork stalling provokes a checkpoint response that hinges on activation of the checkpoint kinase Mec1 (ATR in mammals), and subsequent activation of the Rad53 kinase, the ortholog of the Chk2 tumour suppressor. Mec1 and Rad53 control phosphorylation and activation of the checkpoint kinase Dun1, which shares FHA and kinase domain homology with Rad53 and Chk2 and is required for the full transcriptional response to DNA damage (Zhou and Elledge, 1993; Durocher et al., 2000; Bashkirov et al., 2003). Downstream of Mec1 and parallel to Rad53 and Dun1 lies the checkpoint kinase Chk1, which phosphorylates and stabilizes the anaphase inhibitor Pds1, also known as securin, to inhibit the G2-M transition (Wang et al., 2001). Chk1 plays an unidentified role in the response to replication stress, particularly in cells lacking *DUN1* (Sanchez et al., 1999; Schollaert et al., 2004).

Numerous checkpoint events converge on the RNR complex to promote survival in response to replication stress and to regulate RNR activity during a normal S phase. Two key targets of the Mec1-Rad53-Dun1 cascade have been identified: Sml1, a small physical inhibitor of RNR enzymatic activity (Zhao and Rothstein, 2002), and Crt1, a DNA-binding protein and member of the winged-helix family of transcription factors (Emery et al., 1996). Sml1 is phosphorylated by Dun1 and subsequently targeted for proteolysis, which relieves physical inhibition of RNR activity (Zhao and Rothstein, 2002). Crt1 inhibits transcription at target promoters through recruitment of the general transcriptional repressors Tup1 and Ssn6 (Huang et al., 1998; Li and Reese, 2001). Notably, Crt1 is phosphorylated in a Mec1-Rad53-Dun1-dependent manner after DNA damage or replication stress, thereby promoting its dissociation from promoter DNA and transcriptional activation (Huang et al., 1998). Crt1-repressed targets include the *RNR2*, *RNR3* and *RNR4* genes that encode the DNA-damage-inducible subunits of RNR, *CRT1* itself, as well as *HUG1*, which encodes a small protein that negatively regulates *MEC1*-dependent checkpoint responses (Basrai et al., 1999). Finally, Rnr2 and Rnr4 redistribute from the nucleus to the cytoplasm after DNA damage in a Mec1-Rad53-Dun1-dependent manner (Yao et al., 2003).

The replication checkpoint response slows S-phase progression, stabilizes stalled replication forks, and increases RNR complex activity (Santocanale and Diffley, 1998; Lopes et al., 2001; Tercero and Diffley, 2001; Chabes et al., 2003). Recent data demonstrate that stabilization of replication forks is the main checkpoint function needed for cell survival in the face of replication stress. A collapsed replication fork can lead to DNA double-strand breaks, chromosome rearrangement and loss, and genome instability (Sogo et al., 2002; Tercero et al., 2003). To date, both Mec1 and Rad53 are implicated in stabilization of stalled replication forks (Lopes et al., 2001; Tercero and Diffley, 2001). Mec1-dependent signaling is also required for the fidelity of replication across regions of the genome that are prone to fork stalling and collapse, known as replication slow zones (RSZs) (Cha and Kleckner, 2002) or fragile sites (Lemoine et al., 2005; Admire et al., 2006). These fragile regions appear sensitive to deoxynucleotide levels, reduced DNA polymerase activity and compromised checkpoint function; for example, deletion of *SML1* greatly reduces chromosome breakage at fragile sites in *mec1* mutants (Cha and Kleckner, 2002). There is therefore a crucial link between dNTP metabolism, the replication fork and genome stability.

To identify novel regulators of the cellular response to replication stress, we performed a systematic analysis of 4812 strains in the *S. cerevisiae* haploid non-essential gene deletion set (Giaever et al., 2002) for sensitivity to HU. The screen identified a number of gene deletions that confer HU hypersensitivity, including *CCR4* and *CAF1*/*POP2*. Ccr4 and associated factors called Not proteins are physically and functionally linked to the transcriptional machinery (Denis, 1984; Deluen et al., 2002). Ccr4 is the catalytic component of the major cytoplasmic mRNA deadenylase complex, which regulates poly(A) tail length and influences both mRNA degradation and translation (Tucker et al., 2001; Chen et al., 2002; Tucker et al., 2002). Caf1/Pop2 is a Ccr4-associated factor with suspected nuclease functions (Daugeron et al., 2001). Ccr4 and related protein complexes thus play diverse roles in processes that regulate mRNA abundance and turnover. Here, we show that *CCR4* acts cooperatively with the Mec1-Rad53-Dun1 kinase cascade to help the cell cope with a variety of replication stresses. We identify mutations in *CRT1* as the major suppressor of *ccr4*Δ HU sensitivity and demonstrate that Ccr4 controls *CRT1* mRNA poly(A) tail length and influences Crt1 protein abundance. These results forge a link between mRNA poly(A) metabolism and the tolerance of replication stress.

Results

Ccr4 is a key modifier of the response to DNA replication stress

To identify novel gene activities associated with survival of replication stress, we sought to systematically identify genes required for survival in the presence of HU. We therefore replica-pinned the entire genome-wide set of haploid deletion mutants (Giaever et al., 2002) onto plates containing 100 mM HU. Colony growth was assessed to identify candidate HU-sensitive strains, which were then confirmed in spot tests on 100 mM HU. With this approach, we identified and confirmed 49 HU-sensitive mutants (Table S1 in supplementary material).

While these studies were ongoing, a number of genome-wide phenotypic screens also examined HU sensitivity. In particular, Hartman and Tippery quantitatively profiled arrayed deletion strains for sensitivity to either 50 mM or 150 mM HU (Hartman and Tippery, 2004); Parsons et al. (Parsons et al., 2004) examined arrayed strains robotically pinned to solid media containing 100 mM HU; Bennett et al. (Bennett et al., 2001) identified diploid deletion strains cross-sensitive to both ionizing radiation (primary screen) and 100 mM HU (secondary screen). We compared our confirmed HU-sensitive deletion strains (i.e. hits from genome-wide approach corroborated by spot assay) to the results of other genome-wide studies (Fig. 1A and Table 1). Together, the compiled studies identify 118 common (i.e. found by two or more screens) and 216 unique (i.e. found by only one method) gene deletions that confer HU sensitivity (Fig. 1A). The intersection of all four screens revealed 14 HU-sensitive deletion mutants. A total of 49 high-confidence hits identified in at least three out of four

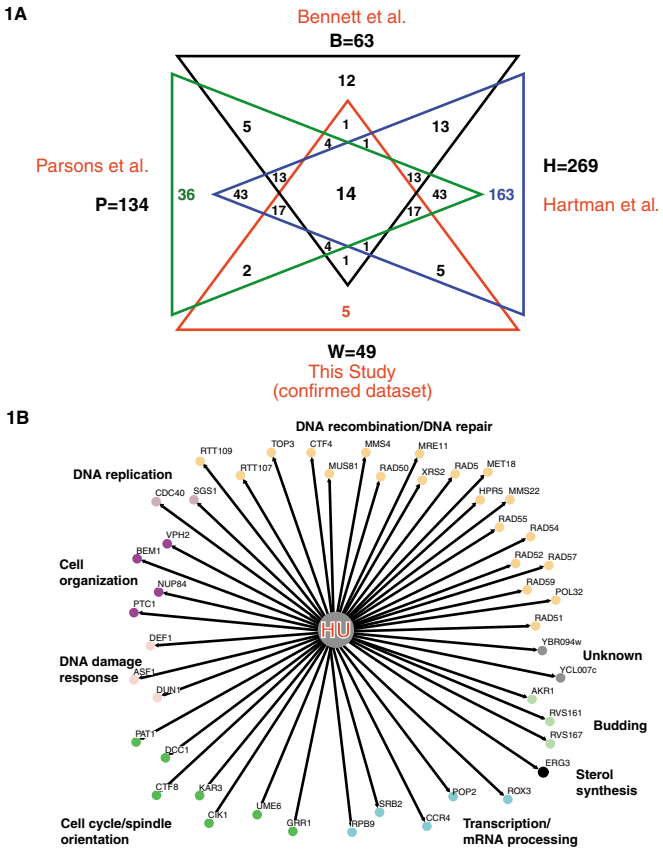


Fig. 1. Multiple genome-wide screens identify common genes required for tolerance to replication stress. (A) Gene deletion strains hypersensitive to hydroxyurea (HU) identified by the approaches of Bennett et al., Hartman and Tippery, Parsons et al. and this study were compiled to identify the shared HU sensitive strains (Bennett et al., 2001; Hartman and Tippery, 2004; Parsons et al., 2004). Number of gene deletions shared between datasets (intersections) or unique to each individual dataset (peripheries) are indicated. (B) HU-sensitive gene deletion mutants identified by at least three of the four approaches were classified by Gene Ontology (GO) process according to the Biological General Repository for Interaction Datasets (BioGRID; www.thebiogrid.org) (Stark et al., 2006). The network was created with Osprey visualization software (Breitkreutz et al., 2003).

Table 1. Genome-wide approaches mutually identify ORF deletions that confer sensitivity to HU

ORF	Gene	Bennett et al. (2001)	Hartman and Tippery (2004)	Parsons et al. (2004)	This study	Overlap‡	GO classification
YAL021c	*CCR4*	+	+	+	+	4	Transcription/mRNA catabolism
YCL016c	*DCC1*	+	+	+	+	4	Sister chromatid cohesion
YDR004w	*RAD57*	+	+	+	+	4	DNA recombination
YDR076w	*RAD55*	+	+	+	+	4	DNA recombination
YDR369c	*XRS2*	+	+	+	+	4	Double-strand break repair
YDR386w	*MUS81*	+	+	+	+	4	DNA repair
YER095w	*RAD51*	+	+	+	+	4	DNA repair
YJR043c	*POL32*	+	+	+	+	4	DNA replication
YLR235c	*TOP3 3′*	+	+	+	+	4	DNA recombination
YLR320w	*MMS22*	+	+	+	+	4	Double-strand break repair
YML032c	*RAD52*	+	+	+	+	4	DNA recombination
YMR190c	*SGS1*	+	+	+	+	4	DNA replication
YNL250w	*RAD50*	+	+	+	+	4	Double-strand break repair
YPR135w	*CTF4*	+	+	+	+	4	DNA replication
YBL093c	*ROX3*		+	+	+	3	RNA pol II transcription
YBR094w			+	+	+	3	Unknown
YBR098w	*MMS4**		+	+	+	3	DNA repair
YBR100w	*MMS4-B**		+	+	+	3	DNA repair
YCR009c	*RVS161*	+		+	+	3	Bud site selection
YCR077c	*PAT1*	+		+	+	3	RNA processing
YDL006w	*PTC1*		+	+	+	3	Protein dephosphorylation
YDL059c	*RAD59*		+	+	+	3	Double-strand break repair
YDL101c	*DUN1*		+	+	+	3	Cell cycle checkpoint
YDR264c	*AKR1*	+		+	+	3	Mating pathway
YDR364c	*CDC40*	+	+		+	3	mRNA splicing
YGL163c	*RAD54*	+	+	+	+	3	Chromatin remodelling
YHR041c	*SRB2*		+	+	+	3	RNA pol II transcription
YHR154w	*ESC4/RTT107*		+	+	+	3	Double-strand break repair
YIL128w	*MET18*		+	+	+	3	RNA pol II transcription
YJR090c	*GRR1*	+		+	+	3	Cell cycle transition
YLR032w	*RAD5*		+	+	+	3	DNA repair
YLR234w	*TOP3*		+	+	+	3	DNA recombination
YMR198w	*CIK1*		+	+	+	3	Mitotic spindle orientation
YMR224c	*MRE11*		+	+	+	3	Double-strand break repair
YNR052c	*CAF1/POP2*		+	+	+	3	Transcription/mRNA catabolism
YPR141c	*KAR3*		+	+	+	3	Mitotic spindle orientation
YBR200w	*BEM1*	+	+	+		3	Cellular polarity
YCL007c		+	+	+		3	Unknown
YDL116w	*NUP84*	+	+	+		3	Nuclear pore complex
YDR207c	*UME6*	+	+	+		3	Transcription
YDR388w	*RVS167*	+	+	+		3	Bud site selection
YGL070c	*RPB9*	+	+	+		3	RNA pol II transcription
YHR191c	*CTF8*	+	+	+		3	Sister chromatid cohesion
YJL092w	*HPR5*	+	+	+		3	DNA repair
YJL115w	*ASF1*	+	+	+		3	Chromatin assembly complex
YKL054c	*DEF1*	+	+	+		3	RNA pol II degradation
YKL119c	*VPH2*	+	+	+		3	Vacuolar acidification
YLR056w	*ERG3*	+	+	+		3	Sterol synthesis

*YBR098w and YBR100w are merged ORFs; ‡sum of studies that identified the particular ORF.
+, identified in the particular study.

screens were classified by Gene Ontology (GO) process (Table 1). As shown in Fig. 1B, many of these mutants are defective in DNA replication, recombination or repair, as expected. However, diverse cellular processes such as transcription, mRNA processing, spindle dynamics, and sister chromatid cohesion were also represented within the intersection dataset. Interestingly, tolerance to HU requires *KAR3* and *CIK1*, which encode proteins that physically interact to form a minus-end-directed kinesin (Chu et al., 2005; Sproul et al., 2005), consistent with recent studies that implicate Mec1 and Rad53 in the regulation of spindle dynamics (Krishnan et al., 2004; Bachant et al., 2005). These genome-wide screens reveal that resistance to replication stress engages diverse cell regulatory processes.

Ccr4 cooperates with Dun1 to enforce replication fork stability

In yeast, Ccr4 and Caf1/Pop2 physically associate to form the major cytoplasmic mRNA deadenylase complex. Ccr4 is the catalytic component of the deadenylase, and Caf1/Pop2 has suspected nuclease functions (Daugeron et al., 2001; Tucker et al., 2002). All of the genome-wide surveys described above, as well as other approaches, uncovered the HU sensitivity of the *ccr4*Δ strain (Fig. 1B and Table 1) (Westmoreland et al., 2004; Traven et al., 2005). The *caf1*Δ/*pop2*Δ strain scored as HU sensitive in three of the four genome-wide analyses (Fig. 1B and Table 1). As removal of either Ccr4 or Caf1/Pop2 confers HU sensitivity, we sought to further investigate their roles in the survival of replication stress. To assess the role of Ccr4

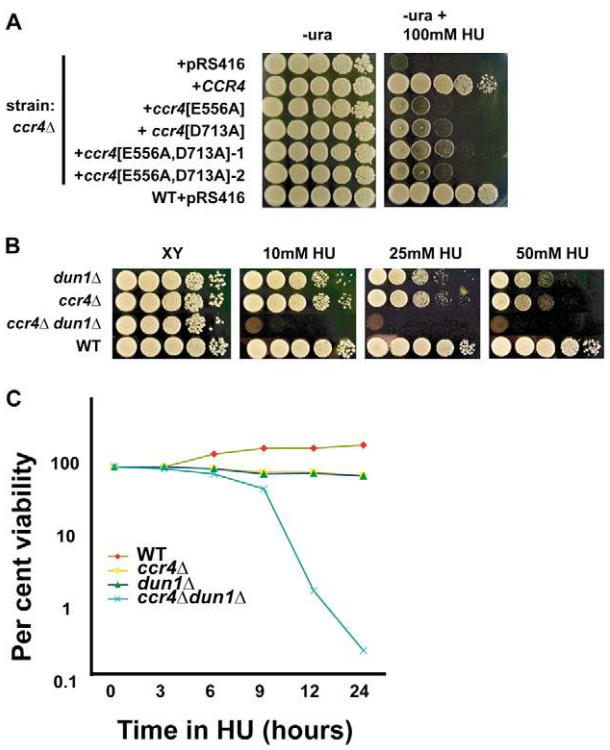


Fig. 2. Ccr4 is required for tolerance to replication stress and acts independently of the Dun1 checkpoint kinase. (A) HU sensitivity of a *ccr4*Δ (MT3768) strain transformed with a pRS416 (CEN) plasmid bearing wild-type *CCR4*; single mutations that abolish in vitro deadenylase activity (*ccr4*[E556A]; *ccr4*[D713A]); or combined double mutations (*ccr4*[E556A, D713A]) were assessed by growth of serial dilutions spotted onto media with or without 100 mM HU for 3 days. (B) Sensitivity of *ccr4*Δ (MT3768), *dun1*Δ (MT3769) or *ccr4*Δ *dun1*Δ (MT3772) strains was analyzed by serial dilution on indicated concentrations of HU as above. (C) Irreversible HU sensitivity was assayed by incubating the indicated strains in liquid media containing 200 mM HU. At indicated time-points, aliquots were removed and cells plated onto rich media lacking HU using a spiral plating system. Colony-forming units (CFU) were determined after incubation for 3 days.

deadenylase activity in the tolerance of replication stress, Ccr4 catalytic residues E556 and D713, which are required for in vitro Ccr4 deadenylase activity, were mutated to alanine (Chen et al., 2002; Tucker et al., 2002). Strains bearing those catalytically compromised *CCR4* alleles were sensitive to 100 mM HU, although less so than the full *CCR4* deletion (Fig. 2A). Deadenylase-dependent and -independent activities of Ccr4 may thus contribute to survival of replication stress or DNA damage.

Since their role in the tolerance to replication stress had not been characterized, we first assessed a possible genetic role for *CCR4* in known replication stress pathways. Deletion of *CCR4* in combination with deletion of the Dun1 checkpoint kinase resulted in dramatic hypersensitivity to HU (Fig. 2B). In particular, the *ccr4*Δ *dun1*Δ strain was sensitive at 10 mM HU, whereas single mutants were only mildly sensitive to HU concentrations above 50-100 mM. The acute sensitivity of the *ccr4*Δ *dun1*Δ strain suggested that the mutant may be defective in stabilization of DNA replication forks, a phenotype observed in *mec1* or *rad53* checkpoint mutants (Lopes et al., 2001; Tercero and Diffley, 2001). We therefore examined whether *ccr4*Δ *dun1*Δ strains were irreversibly sensitive to HU (Allen et al., 1994), a phenotype associated with replication fork collapse (Branzei and Foiani, 2005; Tourriere et al., 2005). Each of the wild-type and single mutant strains tolerated an acute exposure to HU and formed colonies on medium lacking drug, whereas less than 1% of the *ccr4*Δ *dun1*Δ starting cell population was viable after exposure to HU for a 24-hour period, indicating irreversible HU sensitivity (Fig. 2C). These results suggest that Ccr4 and Dun1 collaborate to prevent catastrophic replication fork collapse following HU treatment.

In addition to irreversible HU sensitivity, replication fork collapse produces a potent replication checkpoint signal that persists after the removal of the replication stress (Tercero et al., 2003). Thus, we assessed the activation status of Rad53 in wild-type, *ccr4*Δ, *dun1*Δ and *ccr4*Δ *dun1*Δ strains both during and following acute HU treatment. Rad53 activation was observed in all strains upon addition of HU, as judged by an increase in Rad53 autophosphorylation in kinase assays performed on a membrane blot after in situ re-naturation (Fig. 3A, upper panels). The increase in Rad53 kinase activity was also evident in the appearance of lower mobility Rad53 phospho-isoforms on an anti-Rad53 immunoblot (Fig. 3A, lower panel). Compared with the wild-type control, in each of the *ccr4*Δ and *ccr4*Δ *dun1*Δ strains, the addition of HU elicited a further increase in the magnitude of Rad53 activation, as demonstrated by both Rad53 autophosphorylation and Rad53 mobility shift. The absence of Ccr4 thus enhanced the checkpoint signal during replication stress. Removal of HU from the wild-type cell culture resulted in rapid elimination of slower-migrating forms of phosphorylated Rad53 species, with a concomitant reduction in Rad53 kinase activity (Fig. 3A). By contrast, Rad53 dephosphorylation was delayed in the single mutant *dun1*Δ or *ccr4*Δ cells after removal of HU; this delay was exacerbated in the *ccr4*Δ *dun1*Δ strain (Fig. 3A). These observations suggested persistent checkpoint activation resulting from collapsed replication forks in the *ccr4*Δ *dun1*Δ strain. We conclude that both Ccr4 and Dun1 cooperate to promote replication fork stability in response to nucleotide depletion.

The prolonged Rad53 checkpoint kinase activation upon removal of HU in each of the *ccr4*Δ, *dun1*Δ and *ccr4*Δ *dun1*Δ strains suggested that these strains might exhibit a delay in recovering from replication stress. We therefore analyzed the DNA content of each strain after release from an HU-induced cell-cycle arrest. As expected, the wild-type, *ccr4*Δ, *dun1*Δ, and *ccr4*Δ *dun1*Δ strains exhibited an early S-phase arrest in 0.2 M HU, reflecting a lack of DNA synthesis after early origin firing (Fig. 3B). Consistent with the deactivation kinetics observed for Rad53, the wild-type strain resumed cell-cycle progression approximately 15 minutes after removal of HU, with G2-M phase DNA content readily evident at 45 minutes post release. By contrast, each of the *ccr4*Δ and *dun1*Δ strains were delayed for resumption of cell-cycle progression after release from HU, with significant G2-M DNA content appearing only at 60 minutes post release. The delay in cell-cycle progression was greatly exacerbated in the *ccr4*Δ *dun1*Δ double mutant, which has a reduced G2-M phase DNA content even at 75 minutes post release. Together these observations indicate that Ccr4 and Dun1

cooperate to enable resumption of DNA replication after a period of dNTP shortage.

CCR4 and *CHK1* might act in a genetic pathway for tolerance of DNA replication stress

To further characterize the pathway by which *CCR4* acts to promote tolerance to replication stress, we tested for genetic interactions between *CCR4* and genes encoding components of the replication checkpoint, replication fork machinery and replication apparatus. As the RNR enzyme complex is the molecular target of HU, we reasoned that genetic impairment of RNR activity would hamper growth of the *ccr4*Δ strain. Although *RNR1* and *RNR4* are essential genes in some genetic backgrounds, it is possible to delete either gene in other strain backgrounds, including in the BY4741 background used in this study; such *rnr1*Δ and *rnr4*Δ strains grow slowly and are hypersensitive to HU (Wang et al., 1997; Dubacq et al., 2004). We were therefore able to determine whether cells that lack Ccr4 have a genetic requirement for full RNR activity. Despite the viability of single mutant strains, we were unable to recover strains that bore both *ccr4*Δ and either of the *rnr1*Δ or *rnr4*Δ mutations (Fig. 4A). The inviability of the *ccr4*Δ *rnr4*Δ double mutant was rescued by expression of *RNR4* from a heterologous promoter (data not shown).

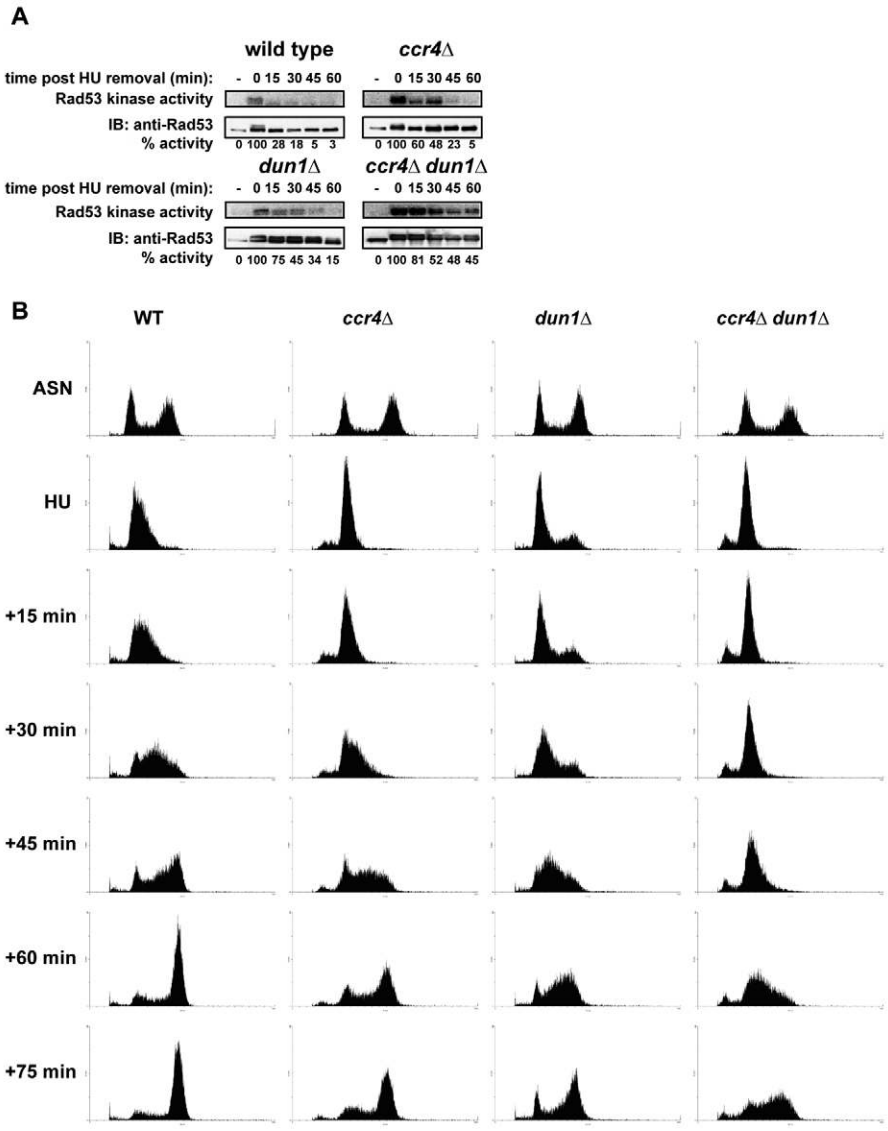


Fig. 3. Rad53 activity persists after HU exposure in cells lacking both *CCR4* and *DUN1*. (A) Wild-type (BY4741), *ccr4*Δ (MT3768), *dun1*Δ (MT3769) and *ccr4*Δ *dun1*Δ (MT3772) strains were treated with 200 mM HU for 3 hours followed by assessment of Rad53 kinase activity by in situ assay (ISA), as manifest in Rad53 autophosphorylation in vitro (upper panel), and by the extent of phosphorylation-dependent Rad53 mobility shift, as detected by the DAB001 Rad53 antibody (lower panel). (B) Cell cycle progression after replication stress is severely delayed in a *ccr4*Δ *dun1*Δ strain. DNA content of the strains in A was determined by FACS analysis of either asynchronous mid-log phase cultures (ASN) or after S-phase synchronization in 200 mM HU for 3 hours (+HU) followed by washout of HU for the indicated times.

We next tested whether the *ccr4*Δ mutation displayed synthetic HU sensitivity in combination with each of the *pol32*Δ, *mrc1*Δ, *rad27*Δ, *tel1*Δ and *elg1*Δ mutations. Each of these deletion strains is defective in replication and/or checkpoint function but has little or no observable growth phenotype in the absence of replication stress. Deletion of *CCR4* in combination with each of the above mutations resulted in a synthetic HU sensitivity phenotype (Fig. 4B). These interactions included marked synthetic HU sensitivity of *ccr4*Δ in combination with the *tel1*Δ and *elg1*Δ mutations, which on their own are largely insensitive to HU (Morrow et al., 1995; Kanellis et al., 2003). Finally, since the DNA polymerase α-primase complex is linked to the DNA damage response, we tested whether deletion of *CCR4* exacerbated the phenotype of a *pri1*-M4 temperature sensitive allele of the *PRI1* gene that encodes the p48 component of the α-primase complex (Marini et al., 1997). Deletion of *CCR4* reduced the permissive temperature of the *pri1-M4* mutation strain from 30°C to 25°C (Fig. 4B). Taken together, these genetic interactions indicate that Ccr4 plays a role in both general DNA replication and in tolerance of replication stress, consistent with related phenotypes observed by Traven et al. (Traven et al., 2005).

Our observation that the *ccr4*Δ *dun1*Δ double mutant strain is highly sensitive to HU indicated that Ccr4 contributes Dun1-independent functions to the tolerance of replication stress. We therefore tested whether the *ccr4*Δ mutation interacted genetically with mutations in other replication checkpoint kinases. The *ccr4*Δ mutation was combined with mutations in *MEC1* (*mec1-21* or the catalytically inactive *mec1*-[*D2224A*] allele), *RAD53* (*sad1-1*) (Allen et al., 1994) or *CHK1*

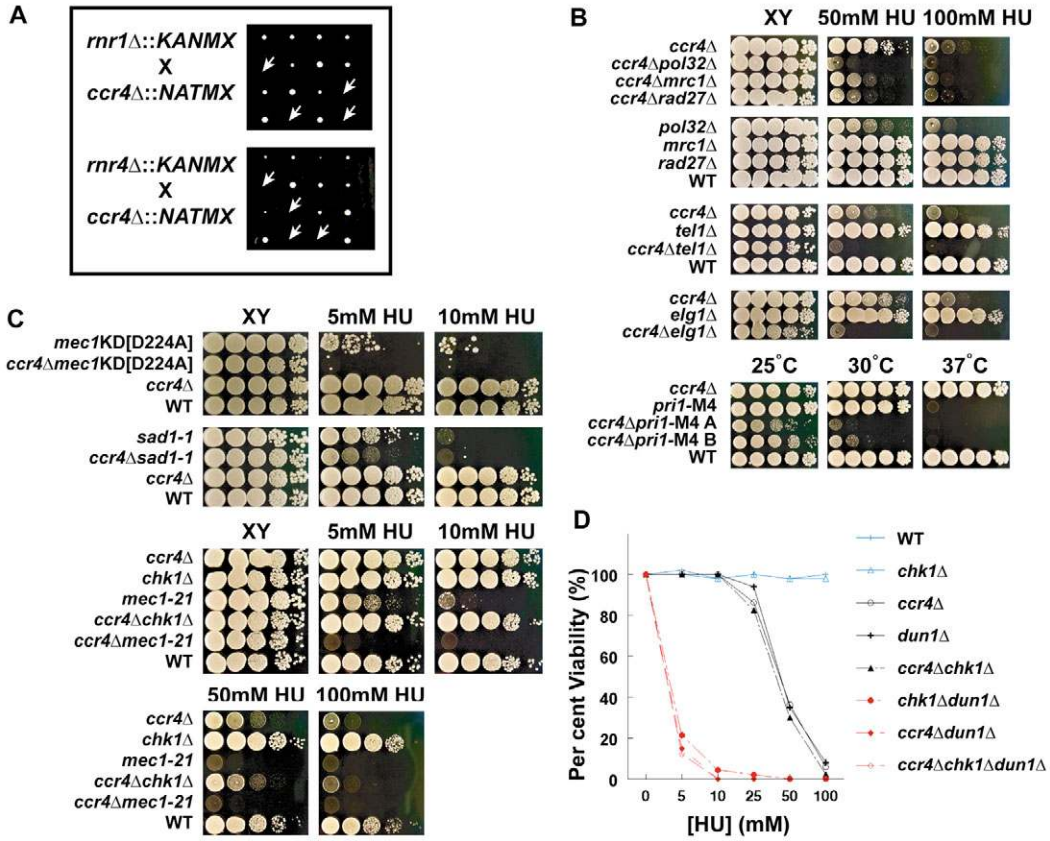


Fig. 4. Genetic interactions between *CCR4*, the replication checkpoint and the DNA replication machinery. (A) Deletion of *CCR4* is synthetic lethal with loss of *RNR1* or *RNR4*. The diploid strains MT3802 (*MAT***a**/α *ccr4*Δ *rnr4*Δ) and MT3809 (*MAT***a**/α *ccr4*Δ *rnr1*Δ) were sporulated, dissected and genotyped (27 tetrads for *ccr4*Δ/*rnr1*Δ cross and 36 tetrads for *ccr4*Δ/*rnr4*Δ cross). Synthetic lethal interactions (*ccr4*Δ *rnr1*Δ or *ccr4*Δ *rnr4*Δ) were inferred from antibiotic resistance marker segregation (arrows). At least one isolate for each possible genotype is depicted. (B) *ccr4*Δ exacerbates the phenotypes of checkpoint and replication mutants. HU sensitivity of the indicated mutants was assessed by serial dilution on media containing indicated concentrations of HU, followed by growth for 3 days. Growth of *pri1*-M4 and *ccr4*Δ *pri1*-M4 (MT3799) strains was assessed on rich media, followed by incubation for 3 days at the indicated temperatures. (C) *CCR4* acts independently of Rad53 and Mec1, but may act in concert with *CHK1*. The indicated checkpoint kinase mutations and strains were analyzed for HU sensitivity as above. (D) Quantitative assessment of sensitivity of the indicated strains to the indicated concentrations of HU was determined with a spiral plating system and CFU determined after 3-5 days growth.

(*chk1*Δ). Not unexpectedly, the HU sensitivity of *mec1* and *rad53* (*sad1*) mutant strains was exacerbated by the absence of Ccr4 (Fig. 4C). Chk1 is part of a less well-characterized branch of the checkpoint response that lies parallel to Rad53 and Dun1 (Sanchez et al., 1999). Although Chk1 plays a crucial role in response to replication stress in fission yeast and in human cells, in budding yeast its role is only revealed when the Rad53-Dun1 branch is disabled; thus, *chk1*Δ *dun1*Δ strains are exceptionally sensitive to HU (Schollaert et al., 2004). As the synthetic HU sensitivity of the *chk1*Δ *dun1*Δ strain is similar to that of the *ccr4*Δ *dun1*Δ strain, we generated *ccr4*Δ *chk1*Δ, *chk1*Δ *dun1*Δ and *ccr4*Δ *chk1*Δ *dun1*Δ mutant strains to assess whether *CHK1* lies in the same genetic pathway as *CCR4*. We observed that the *ccr4*Δ *chk1*Δ double mutant was no more sensitive to HU than the *ccr4*Δ mutant and that the *ccr4*Δ *chk1*Δ *dun1*Δ triple mutant strain had a similar HU sensitivity to the *ccr4*Δ *dun1*Δ strain (Fig. 4C,D). These epistatic genetic relationships suggest that *CHK1* and *CCR4* may function together in a novel genetic pathway that contributes to the tolerance of replication stress.

Deletion of either *CRT1* or *PBP1* suppresses HU lethality of the *ccr4*Δ mutant

Since Ccr4 deadenylase activity is thought to negatively regulate gene expression, we reasoned that deletion of potential Ccr4 targets might suppress the HU sensitivity of *ccr4*Δ strains. To identify potential suppressors of the *ccr4*Δ HU sensitivity we undertook an insertional mutagenesis screen in a *ccr4*Δ strain with a genomic transposon (Tn3-*LEU2*) library (Ross-Macdonald et al., 1997). From a pool of 300,000 Tn3-*LEU2* transformants, we obtained 30 single colony suppressors of the *ccr4*Δ growth defect on HU. Sequencing of the transposon insertion sites identified 24 candidate suppressor genes, of which ten independent genes accounted for the entire suppressor pool (Table 2). The two strongest suppressors were *crt1*::Tn3-*LEU2* (eight occurrences) and *pbp1*::Tn3-*LEU2* (six occurrences). *CRT1* encodes the major transcriptional repressor of DNA-damage-regulated genes, whereas *PBP1* encodes a factor that interacts with Pab1, the major poly(A) binding protein in budding yeast, and is believed to regulate polyadenylation (Mangus et al., 1998). To confirm the strongest

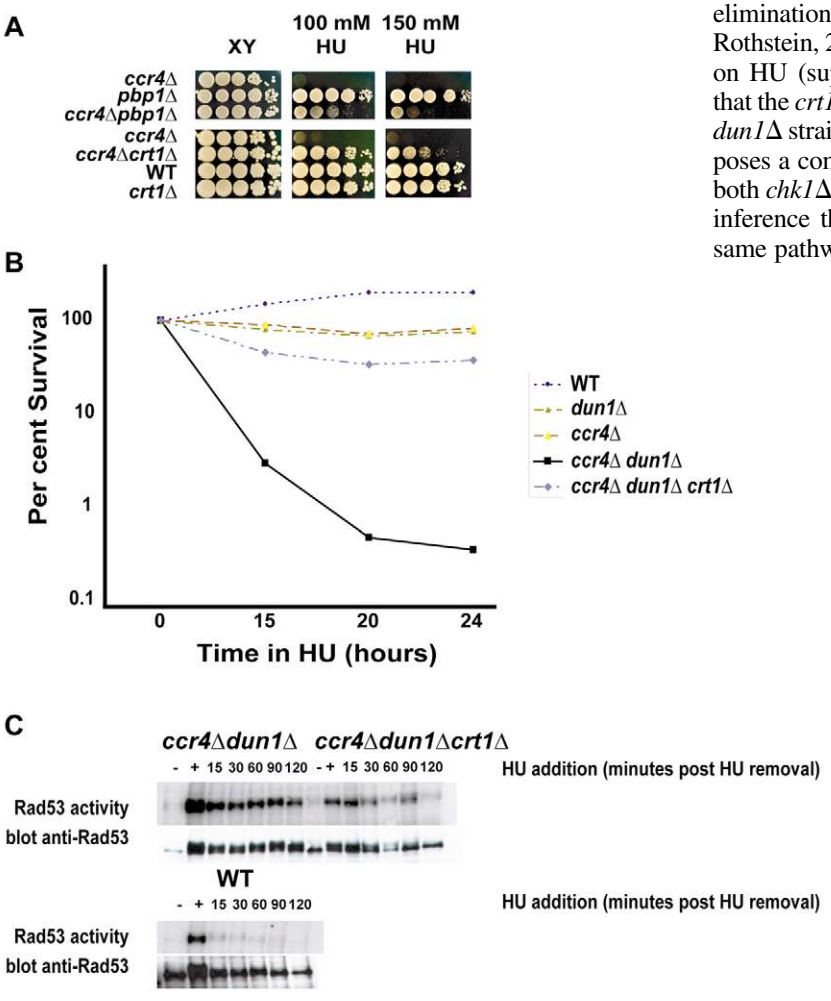
Table 2. An insertional mutagenesis screen reveals *ORF* disruptions that suppress HU lethality of the *ccr4*Δ strain

Gene	ORF	Occurrence in suppressor pool*	Suppression of *ccr4*Δ phenotype corroborated by independent co-deletion?†	Characteristics of encoded protein
CRT1	YLR176c	8/24	+++	Transcriptional repressor of DNA damage regulon
PBP1	YGR178c	6/24	++	Pab1-binding protein; polyadenylation factor
MOT1	YPL082c	2/24	ND (essential gene)	Transcriptional regulator; putative helicase
GTR1	YML121w	2/24	ND (very slow growth)	Small GTPase; phosphate regulation
TIF2	YJL138c	1/24	–	Translation initiation factor eIF4A
TUP1	YCR084c	1/24	+/–	General transcriptional repressor
ECM29	YHL030w	1/24	–	Major component of proteasome
HXT8	YJL214w	1/24	–	Similar to hexose transporters
PHO86	YJL117w	1/24	–	Regulation of phosphate transporter Pho84
YCR076c	YCR076c	1/24	–	Unknown

*Number of times identified among 24 suppressors from initial pool of 300,000 transformants; †suppression of HU lethality ranked on a scale from none (–) to very strong (+++).
ND, not determined.

hits from the suppressor screen, independent deletions of *CRT1* and *PBP1* were constructed in the context of either *ccr4*Δ or *ccr4*Δ *dun1*Δ strains. Deletion of *CRT1* suppressed not only the HU lethality of a *ccr4*Δ strain but also of a *ccr4*Δ *dun1*Δ mutant (Fig. 5A and supplementary material Fig. S1A). An independent deletion of *PBP1* also suppressed the HU lethality of a *ccr4*Δ strain, although less so than *crt1*Δ (Fig. 5A).

We then tested whether deletion of *CRT1* also suppressed the severe and irreversible hypersensitivity to HU of the *ccr4*Δ *dun1*Δ strain. The *ccr4*Δ *dun1*Δ *crt1*Δ triple mutant strain retained viability even after 24-hour exposure to HU (Fig. 5B). Consistent with this bypass effect, Rad53 activity did not reach the same magnitude of induction in a *ccr4*Δ *dun1*Δ *crt1*Δ strain compared with a *ccr4*Δ *dun1*Δ strain, although activity did still persist at prolonged time points after HU withdrawal. The incomplete bypass and the residual checkpoint activation in the *ccr4*Δ *dun1*Δ *crt1*Δ strain suggested that Ccr4 and/or Dun1 have additional targets that affect checkpoint recovery. However, elimination of the characterized Dun1 target Sml1 (Zhao and Rothstein, 2002) had little effect on *ccr4*Δ *dun1*Δ strain viability on HU (supplementary material Fig. S1A). We also observed that the *crt1*Δ mutation suppressed the HU sensitivity of a *chk1*Δ *dun1*Δ strain (supplementary material Fig. S1B). *CRT1* therefore poses a common impediment to survival of replication stress in both *chk1*Δ *dun1*Δ and *ccr4*Δ *dun1*Δ strains, consistent with the inference that *CHK1* and *CCR4* appear to act together in the same pathway.



Ccr4 regulates *CRT1* mRNA poly(A) tail length and influences Crt1 protein level

Based on the above observations, we postulated that in the absence of Ccr4 the *CRT1* mRNA is deregulated, which subsequently leads to HU sensitivity. To identify potential alterations in *CRT1* mRNA poly(A) tail length, wild type, *ccr4*Δ or *ccr4-1* (i.e. the catalytic mutant allele, E556A) strains were treated or not with 100 mM HU for 90 minutes and then

Fig. 5. Deletion of *CRT1* or *PBP1* suppresses the HU sensitivity of a *ccr4*Δ strain. (A) *ccr4*Δ *crt1*Δ (MT3771), *ccr4*Δ *pbp1*Δ (MT3777), wild-type (BY4741), *ccr4*Δ (MT3768), *crt1*Δ (MT3770) and *pbp1*Δ (MT3776) strains were tested for HU sensitivity by serial dilution analysis, as described in Fig. 2. (B) Indicated strains were assessed for irreversible HU sensitivity using a spiral plating system, as described in Fig. 2C. (C) Rad53 activity and phosphorylation were assessed in *ccr4*Δ *dun1*Δ (MT3772), *ccr4*Δ *dun1*Δ *crt1*Δ (RSYS07) and wild-type (BY4741) strains after exposure to 0.2 M HU for 3 hours, as described for Fig. 3.

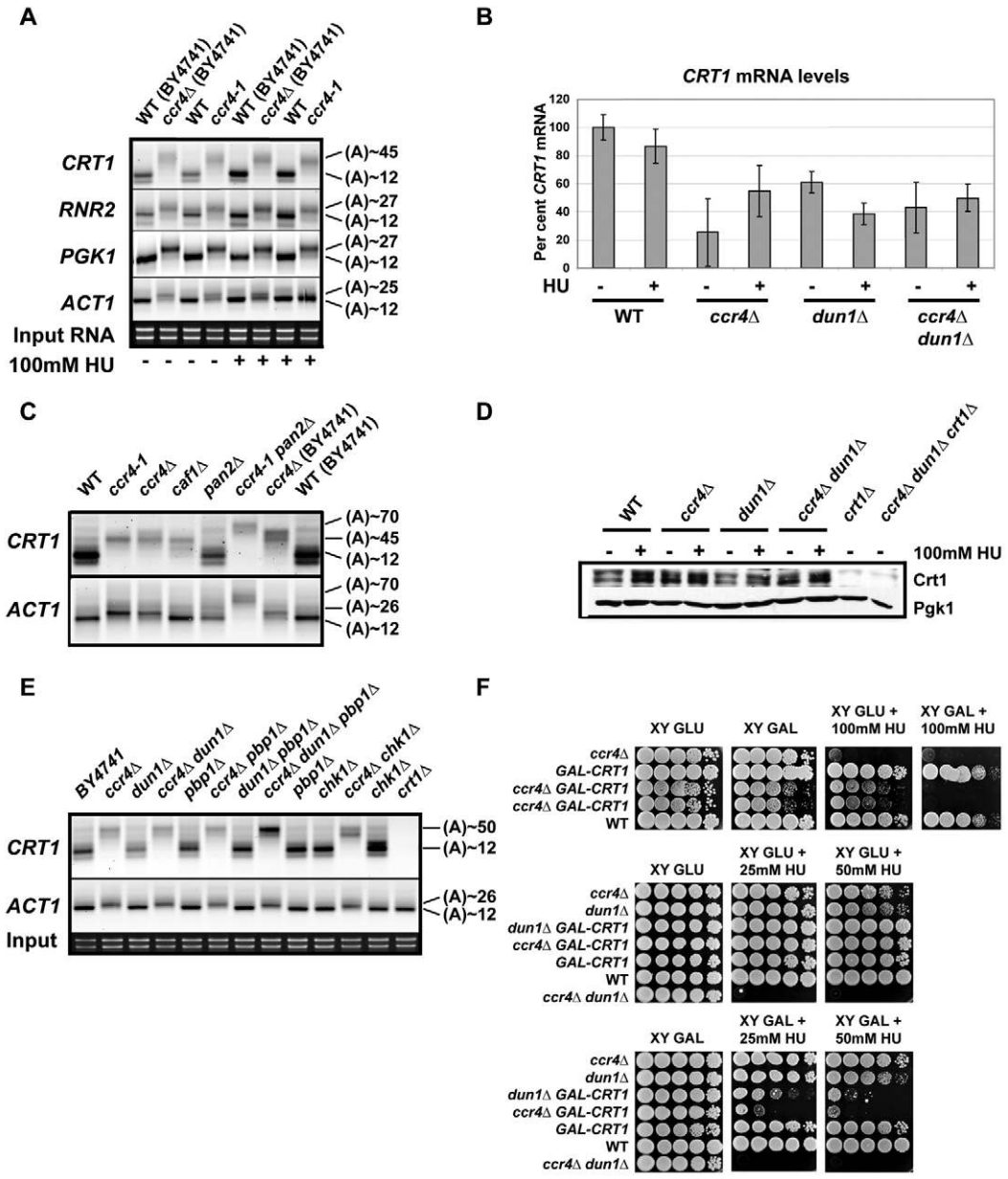


Fig. 6. Ccr4 regulates *CRT1* mRNA poly(A) tail length. (A) Poly(A) tail lengths were determined by LM-PAT analysis of total cellular RNA isolated from isogenic *ccr4*Δ (MT3768) and wild-type (BY4741), and from isogenic *ccr4-1* (Y359) and wild-type (Y136) strain pairs, each either untreated or treated with 100 mM HU for 90 minutes. The estimated number of adenosine residues in the poly(A) tails from *ccr4*Δ strains are noted for specific transcripts. Notice that a segment of 12 adenosines is introduced by the linker primer into all cDNA produced, and thus this length represents the lowest limit of PCR product size from short tailed mRNAs. (B) *CRT1* mRNA levels were determined by quantitative Real Time PCR of total RNA isolated from wild-type (BY4741), *ccr4*Δ (MT3768), *dun1*Δ (MT3769) or *ccr4*Δ *dun1*Δ (MT3772) strains that were either untreated or treated with 100 mM HU for 90 minutes. *CRT1* mRNA levels were normalized to *ACT1* mRNA levels and reported as the fraction of untreated wild-type *CRT1* mRNA abundance. (C) Alteration of *CRT1* mRNA poly(A) tail length in wild-type (Y136), *ccr4-1* (Y359), *ccr4*Δ (Y294), *caf1*Δ (Y297), *pan2*Δ (YTP1), *ccr4-1 pan2*Δ (YTP2), *ccr4*Δ (MT3768) and wild-type (BY4741) strains by LM-PAT analysis of total RNA. (D) Crt1 protein abundance was determined in wild-type (BY4741), *ccr4*Δ (MT3768), *dun1*Δ (MT3769) and *ccr4*Δ *dun1*Δ (MT3772) strains that were either untreated or treated with 100 mM HU for 90 minutes. Crt1 was detected with polyclonal anti-Crt1 antibody. Strains *crt1*Δ (MT3770) and *ccr4*Δ *dun1*Δ *crt1*Δ (MT3773) served as negative controls for antibody specificity; equivalent protein loading was assessed with a monoclonal anti-Pgk1 antibody. (E) *CRT1* mRNA poly(A) tail lengths were determined in wild-type (BY4741), *ccr4*Δ (MT3768), *dun1*Δ (MT3769), *ccr4*Δ *dun1*Δ (MT3772), *pbp1*Δ (MT3776), *ccr4*Δ *pbp1*Δ (MT3777), *dun1*Δ *pbp1*Δ (MT3842), *ccr4*Δ *dun1*Δ *pbp1*Δ (MT3843), *chk1*Δ (MT3784) and *ccr4*Δ *chk1*Δ (MT3785) strains by LM-PAT analysis of total RNA. The number of adenosine residues for major poly(A) tail species for specific transcripts are indicated. (F) The effect of *CRT1* overexpression on tolerance to replication stress was determined with an integrated *GAL-CRT1* allele in strains *GAL1-CRT1* (MT3806), *ccr4*Δ *GAL1-CRT1* (MT3807) and *dun1*Δ *GAL1-CRT1* (MT3808). Sensitivity to HU was determined by serial dilution and spotting on indicated concentrations of HU under conditions of *GAL1-CRT1* repression (+GLU) or induction (+GAL). Wild-type (BY4741), *ccr4*Δ (MT3768), *dun1*Δ (MT3769) and *ccr4*Δ *dun1*Δ (MT3772) strains were used as controls.

various mRNAs analyzed for poly(A) tail length using a ligase-mediated poly(A) (LM-PAT) assay (Salles et al., 1999). Compared with the wild type control strain, *CRT1* mRNA poly(A) tails were substantially longer in the *ccr4*Δ and *ccr4-1* strains (Fig. 6A). The poly(A) tail length of other mRNAs, including *RNR2*, *PGK1* and *ACT1* were also noticeably elongated in the *ccr4* mutants. However, *CRT1* mRNA had the longest poly(A) tail of all mRNAs examined in the *ccr4*Δ strain at 45 adenosine residues, compared with 12 adenosines residues in a wild-type strain (Fig. 6A). A known Ccr4 substrate, the *PGK1* mRNA, had a poly(A) tail length of 27 adenosine residues in the *ccr4*Δ strain; *RNR2* and *ACT1* poly(A) tails had lengths of 27 and 22 adenosine residues, respectively. HU treatment did not appreciably affect poly(A) status of any of the transcripts tested. Consistent with the HU sensitivity of the *caf1*Δ/*pop2*Δ strain detected in our genome-wide screen and with the known physical association of Caf1/Pop2 with Ccr4, the *CRT1* mRNA poly(A) tail was also elongated to 45 adenosine residues in a *caf1*Δ strain (Fig. 6C). By contrast, removal of the Pan2 poly(A) nuclease had little effect on *CRT1* mRNA poly(A) tail length. The *CRT1* mRNA poly(A) tail was greatly elongated to 70 adenosine residues in the *ccr4-1 pan2*Δ double mutant strain, in accord with the dual role for the Pan2/Pan3 and Ccr4 nucleases as the major regulators of poly(A) tail length (Tucker et al., 2001).

Deadenylation of the poly(A) tail is implicated in mRNA decay (Parker and Song, 2004) and, moreover, Ccr4 is implicated in transcriptional control as well as mRNA deadenylation (Collart, 1996; Tucker et al., 2001). We therefore used quantitative real time PCR to address the possible effect of *ccr4*Δ on *CRT1* mRNA levels, both in the absence and presence of HU. Contrary to our initial expectations, *CRT1* mRNA abundance in the *ccr4*Δ strain was actually reduced to 25% of that in a wild-type strain during vegetative growth, with a statistically insignificant increase to 55% after HU treatment (Fig. 6B; all comparisons with untreated wild-type control). *CRT1* mRNA abundance was also lowered in a *dun1*Δ strain to 61% of wild-type levels, consistent with the known role for Dun1 in *CRT1* transcriptional induction (Huang et al., 1998). *CRT1* transcript levels in the *ccr4*Δ *dun1*Δ double mutant strain did not appreciably differ from either single mutant. After HU treatment, *CRT1* mRNA in the *ccr4*Δ *dun1*Δ strain also increased somewhat, to approximately 50% of WT levels. In wild-type cells, we observed little induction of the *CRT1* mRNA in response to HU, a result that is at odds with previous findings that the *CRT1* mRNA is induced 1.9-fold by HU relative to *ACT1* mRNA levels (Huang et al., 1998). This discrepancy may in part derive from the use of different strain backgrounds and different conditions [BY4741 and 100 mM HU in this study, versus W303 and 200 mM HU in previous work (Huang et al., 1998)]. In addition, we noticed that *ACT1* mRNA abundance was consistently increased in response to HU in our wild-type strain, such that possible induction of the *CRT1* mRNA is masked by normalization to *ACT1* (Fig. 6A; T.B. and R.W., unpublished observations). Thus, although it is evident that Ccr4 modulates both the poly(A) tail length and abundance of the *CRT1* mRNA, the observed decrease in *CRT1* mRNA levels is not consistent with the observed genetic hyperactivity of *CRT1* in a *ccr4*Δ strain.

Elongated poly(A) tails are associated with increased rates of translation (Preiss et al., 1998). To address possible countervailing effects of elongated poly(A) tails and concomitantly decreased *CRT1* mRNA in the *ccr4*Δ strain, Crt1 protein levels were assessed with a polyclonal antibody to the N-terminus of Crt1 (Zhang and Reese, 2005). In contrast to the observed drop in *CRT1* mRNA, Crt1 protein was not decreased during vegetative growth in a *ccr4*Δ strain when compared with wild-type controls (Fig. 6D). As expected, in both the wild-type and *ccr4*Δ strains, Crt1 protein levels were moderately increased upon HU treatment. However, HU did not elicit as great an increase in Crt1 in a *dun1*Δ strain, in accord with the known role for Dun1 in *CRT1* transcriptional induction (Huang et al., 1998). Importantly, the induction of Crt1 protein by HU was restored in the *ccr4*Δ *dun1*Δ strain, suggesting that the effect of the *ccr4*Δ mutation is independent of the checkpoint itself. As a control for these experiments, Pgk1 levels were constant in all strains and conditions tested (Fig. 6D). Taken together, the above data suggest complex effects in the *ccr4*Δ strain, including a possible scenario in which failure to deadenylate the *CRT1* mRNA increases translational capacity per *CRT1* mRNA molecule.

As *pbp1* alleles were also uncovered as partial suppressors of the *ccr4*Δ HU lethality, and as Pbp1 is a putative regulator of both polyadenylation and translation, we examined *CRT1* mRNA poly(A) tail length in a panel of gene deletion mutants, including *pbp1*Δ strains (Fig. 6E). However, no difference in *CRT1* mRNA poly(A) tail length between wild-type and the *pbp1*Δ mutant was detected. In addition, no difference in *CRT1* mRNA poly(A) tail length was apparent between *ccr4*Δ and *ccr4*Δ *pbp1*Δ strains. Thus, it does not appear that *pbp1*-mediated suppression of *ccr4*Δ HU lethality occurs at the level of the *CRT1* poly(A) tail. Since Pbp1 is a ribosome-associated factor (Fleischer et al., 2006) and has been previously implicated in 3′ end-dependent translation (Tadauchi et al., 2004), we posit that the suppression of *ccr4*Δ HU sensitivity upon removal of Pbp1 is mediated through decreased translation of the *CRT1* transcript in the *ccr4*Δ *pbp1*Δ strain.

CRT1 overexpression hypersensitizes *ccr4*Δ and *dun1*Δ strains to HU

Our genetic results suggested that deregulated *CRT1* activity may cause severe HU lethality in *ccr4*Δ *dun1*Δ cells. To test this notion, we integrated a *GAL1-CRT1* allele into *ccr4*Δ and *dun1*Δ strains and assessed the HU sensitivity of each strain on repressive and inducing media. As expected, repression of *CRT1* on glucose medium suppressed the HU lethal phenotypes of both *ccr4*Δ and *dun1*Δ strains (Fig. 6F). Conversely, galactose induction of *CRT1* hypersensitized both the *ccr4*Δ and *dun1*Δ strains, such that both were compromised even at low concentrations of HU. Overexpression of *CRT1* had only a modest effect on the HU sensitivity of a wild-type strain, indicating that robust mechanisms normally keep *CRT1* activity in check. These genetic data suggest that *DUN1* and *CCR4* represent two independent genetic pathways that repress *CRT1* activity during replication stress.

Deregulated *RNR* gene transcription contributes to HU sensitivity of *ccr4*Δ strains

Since Crt1 is a transcriptional repressor, we employed genome-wide expression profiles to identify putative Crt1 targets. We compared the transcriptional profiles of *ccr4*Δ and *ccr4*Δ

*dun1*Δ strains to those of analogous strains in which *CRT1* had been deleted, i.e. *ccr4*Δ *crt1*Δ and *ccr4*Δ *dun1*Δ *crt1*Δ strains in competitive hybridizations against genome-wide DNA microarrays (Fig. 7A,B). In each genetic context, deletion of *CRT1* resulted in a dramatic upregulation of its known target genes, including *RNR2, RNR3* and *RNR4* (Fig. 7A,B; Table 3).

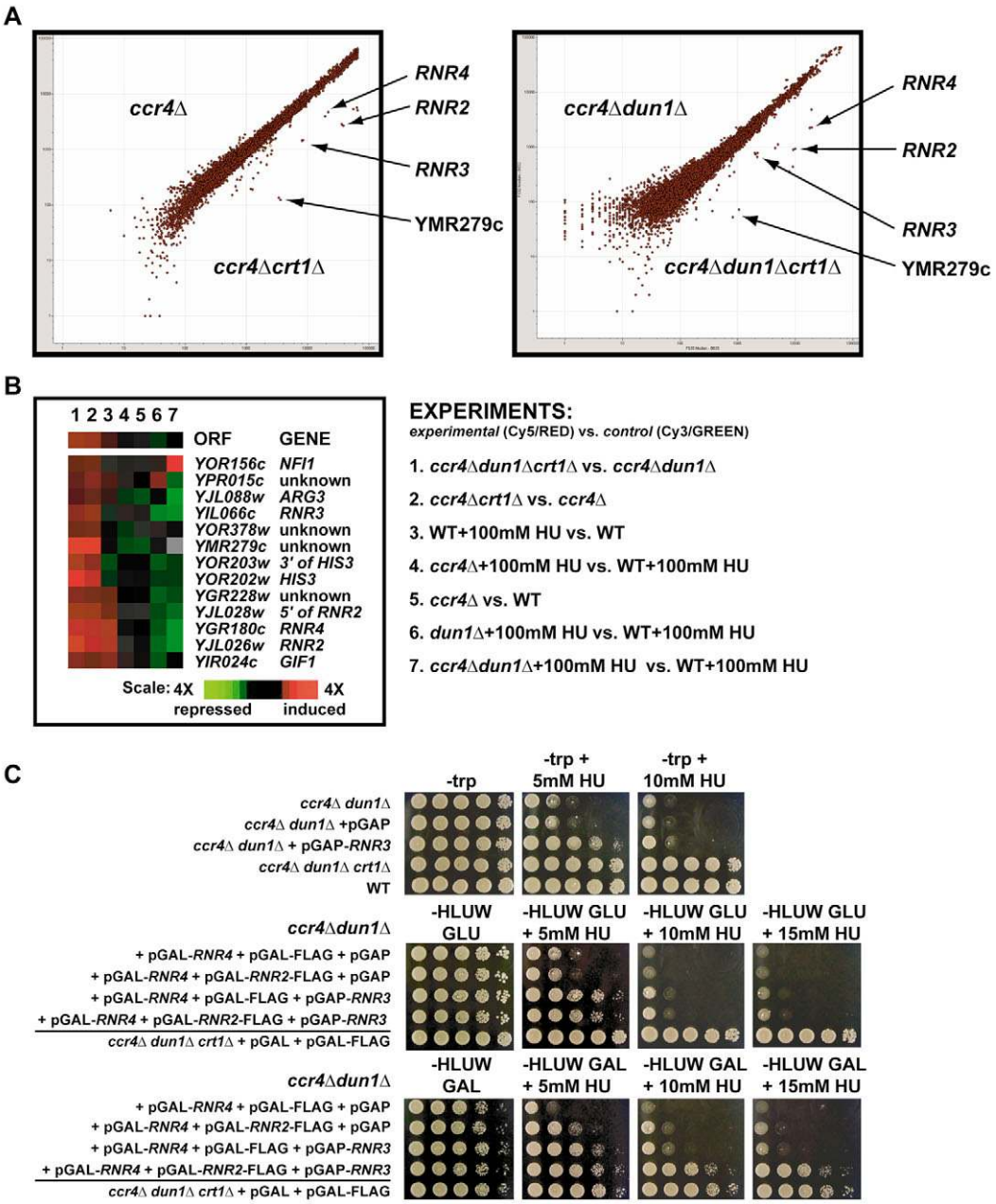


Fig. 7. Crt1 controls transcription of a suite of genes that may affect viability during replication stress. (A) Genome-wide transcriptional profiles were obtained by competitive hybridization of labeled cDNAs from *ccr4*Δ *crt1*Δ (MT3771, Cy5) vs. *ccr4*Δ (MT3768, Cy3) and for *ccr4*Δ *dun1*Δ *crt1*Δ (MT3773, Cy5) vs. *ccr4*Δ *dun1*Δ (MT3772, Cy3), as described in Materials and Methods. See Table 2 for specific gene induction/repression values. (B) Top differentially regulated genes between indicated strains, with and without HU treatment. Scale shows relative induction (red) or repression (green) with respect to control samples. (C) Effect of overexpression of *RNR* genes on sensitivity of the *ccr4*Δ *dun1*Δ double mutant to HU. (Top panels) Overexpression of *RNR3*. Strain *ccr4*Δ *dun1*Δ *trp1*Δ (MT3800) was transformed with either pGAP (pBAD054 *TRP1*, empty) or pGAP-*RNR3* (pBAD079 *TRP1*, *RNR3*). *ccr4*Δ *dun1*Δ (MT3772), *ccr4*Δ *dun1*Δ *crt1*Δ (MT3773) and wild-type (BY4741) strains are shown for comparison. Strains were serially diluted, spotted on media lacking tryptophan (-trp) and containing indicated concentrations of HU and grown for 3-5 days. (Middle and bottom panels) Coordinate overexpression of multiple *RNR* subunits. Strain *ccr4*Δ *dun1*Δ *trp1*Δ (MT3800) was transformed with either pGAP (pBAD054 *TRP1*, empty vector) or pGAP-*RNR3* (pBAD079 *TRP1*, *RNR3*) in combination with pGAL-*RNR4* (pDL57 *URA3*, *RNR4*) and either pGAL-FLAG (pMT3164 *LEU2*, empty vector) or pGAL-*RNR2*-FLAG (pMT3963 *LEU2*, *RNR2*-FLAG). *ccr4*Δ *dun1*Δ *crt1*Δ (MT3773) was co-transformed with pGAL (pDL54 *URA3*, empty vector) or pGAL-FLAG (pMT3164 *LEU2*, empty vector) as a comparison. Strains were serially diluted, spotted on media lacking histidine, leucine, uracil and tryptophan (-HLUW) and containing indicated concentrations of HU and either glucose (GLU) or galactose (GAL) and grown for 3-5 days.

Furthermore, HU-induced transcription of both *RNR2* and *RNR4* was severely impaired in a *ccr4*Δ *dun1*Δ strain, much more so than in either *dun1*Δ or *ccr4*Δ strains (Fig. 7B). Removal of *CRT1* thus enables *ccr4*Δ and *ccr4*Δ *dun1*Δ strains to respond to and cope with replication stress through heightened induction of *RNR* genes. Although it has been previously reported that *RNR3* is induced in a *ccr4*Δ strain upon addition of HU (Traven et al., 2005), our transcriptional profiles did not corroborate this result (Table S2 in supplementary material). A recent direct analysis of *RNR* transcripts in a variety of Ccr4-Not complex mutants concurs with our genome-wide expression profiles (Mulder et al., 2005).

The set of approximately 16 to 37 genes (depending on genetic context) induced more than twofold in the absence of *CRT1* all represent candidate modulators of HU resistance in either a *ccr4*Δ or a *ccr4*Δ *dun1*Δ strain. To date we have been unable to identify any single multi-copy or overexpression suppressor of the *ccr4*Δ defect, in either direct tests with this gene set or in suppression screens with a 2-μm plasmid genomic library (data not shown). As shown in Table 3, the ORF most responsive to *CRT1* deletion was YMR279c, which is also induced by MMS (Gasch et al., 2001). YMR279c putatively encodes a putative 12 transmembrane-spanning protein of unknown function but with homology to drug transporters. However, deletion of YMR279c did not affect sensitivity to HU in any background tested (data not shown).

The above genetic and gene expression data suggested that a severe defect in *RNR* subunit expression might be responsible for HU hypersensitivity of *ccr4*Δ *dun1*Δ cells. To test this idea, *RNR2* or *RNR4* were expressed from the conditional *GAL1* promoter, either alone or in combination with *RNR3* expressed from the constitutive *GAPDH* (*GAP*) promoter. Expression of either *RNR3* alone or co-expression of *RNR2* and *RNR4* together from heterologous promoters only slightly improved the viability of a *ccr4*Δ *dun1*Δ strain on low concentrations of HU, whereas combined expression of *GAP-RNR3* and *GAL1-RNR4* further augmented survival. The combined heterologous expression of *GAL1-RNR2*, *GAP-RNR3* and *GAL1-RNR4* significantly improved viability on HU, although this bypass was still not as strong as with the *crt1*Δ mutation itself (Fig. 7C). In contrast to the partial bypass of the *ccr4*Δ *dun1*Δ strain

Table 3. Transcriptional responses after deletion of *CRT1* in *ccr4*Δ and *ccr4*Δ *dun1*Δ strains

ORF	Gene	Level of induction (repression) in *ccr4*Δ *dun1*Δ *crt1*Δ vs *ccr4*Δ *dun1*Δ (log_2)*	Level of induction (repression) in *ccr4*Δ *crt1*Δ vs *ccr4*Δ (log_2)*
YMR279c		4.6	4.7
YJL026w	*RNR2*	4.1	3.6
YGR180c	*RNR4*	3.7	3.4
YJL028w		2.7	2.6
YOL121c	*RPS19A*	2.6	0.0
YHR056c	*RSC30*	2.2	0.8
YIL066c	*RNR3*	2.2	2.4
YPL256c	*CLN2*	2.1	0.3
YIR024c	*GIF1*	2.0	1.5
YGR228w		2.0	1.7
YLR233c	*EST1*	1.8	0.3
YGL062w	*PYC1*	1.6	(–0.2)
YOR378w		1.5	1.7
YGR206w		1.5	0.1
YOR156c	*NFI1*	1.4	1.6
YLR244c	*MAP1*	1.4	0.1
YPR052c	*NHP6A*	1.4	0.6
YIR013c	*GAT4*	1.3	(–0.1)
YLR329w	*REC102*	1.3	(–0.9)
YMR267w	*PPA2*	1.3	(–0.8)
YPR015c		1.2	2.0
YLR177w		1.2	0.9
YPR007c	*SPO69*	1.2	0.4
YGL182c		1.1	0.2
YNL254c		1.1	0.2
YPL170w	*DAP1*	1.1	1.2
YEL047c		1.1	0.8
YDR255c	*RMD5*	1.1	(–0.4)
YOR225w		1.1	(–2.0)
YPR042c	*PUF2*	1.1	(–1.0)
YNL179c	*SRF6*	1.1	0.0
YNL139c	*RLR1*	1.1	0.1
YJL088w	*ARG3*	1.1	1.3
YLL005c	*SPO75*	1.0	1.0
YER171w	*RAD3*	1.0	0.2
YGL103w	*RPL28*	1.0	0.4
YJR125c	*ENT3*	1.0	0.2
YMR283c	*RIT1*	(–0.2)	1.3
YGL208w	*SIP2*	(–0.4)	1.3
YIL013c	*PDR11*	0.7	1.3

*Experimental (Cy5 label) vs reference (Cy3 label).

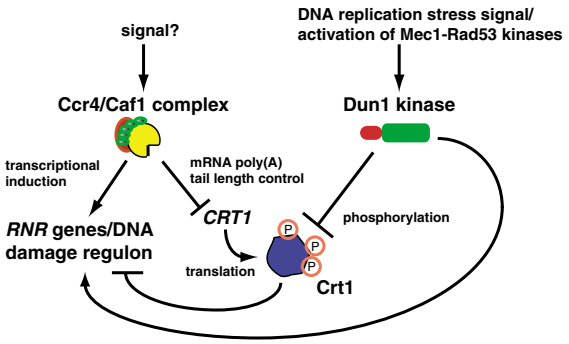


Fig. 8. Ccr4 and Dun1 collaborate to inhibit Crt1 activity and to promote induction of the DNA-damage gene regulon after replication stress. Ccr4 regulates *CRT1* mRNA poly(A) tail length, which may influence translation and thereby levels of Crt1 protein. The Mec1-Rad53-Dun1 checkpoint kinase cascade phosphorylates and inhibits Crt1 repressor activity at DNA-damage-inducible promoters. Loss of either regulatory branch results in HU sensitivity; simultaneous loss of both regulatory branches causes severe and irreversible HU lethality.

HU sensitivity upon heterologous expression of *RNR3*, expression of *RNR1* from the *GAP* promoter failed to improve survival of a *ccr4*Δ *dun1*Δ strain on HU (supplementary material Fig. S1C). These data suggest a defect in coordinated *RNR2*, *RNR3* and *RNR4* transcription, substantially contributing to the observed inviability of the *ccr4*Δ *dun1*Δ strain in the presence of HU. The differential expression of other Crt1 target genes may also contribute to the survival of the *ccr4*Δ *dun1*Δ *crt1*Δ strain on HU.

Discussion

Through a systematic screen of the budding yeast gene deletion collection for HU-sensitive strains we have discovered that Ccr4, the catalytic component of the mRNA deadenylase complex, plays a crucial role in the response to replication stress. Other recent reports have also implicated Ccr4 in resistance to HU-induced replication stress (Bennett et al., 2001; Westmoreland et al., 2004; Traven et al., 2005). Because our genetic analysis shows that *mec1*, *rad53* and *dun1*Δ mutants, but not *chk1Δ* mutants, are hypersensitized to HU upon deletion of *CCR4*, it appears Ccr4 operates in parallel to the Mec1-Rad53-Dun1 pathway to resolve DNA replication stress, perhaps in concert with Chk1. Consistently, we note that *CCR4* and *RAD9* act in the same epistasis group for resistance to ionizing radiation (Westmoreland et al., 2004) and that Rad9 physically interacts with Chk1 after DNA damage (Sanchez et al., 1999); together with our findings, these results suggest that Chk1 and Ccr4 act in the same branch of the replication checkpoint.

Our genetic and biochemical analysis implicates Ccr4 in the regulation of Crt1 function, and suggests that Crt1-mediated repression of transcription poses a major impediment to survival after exposure to HU. Crt1 activity is known to be negatively regulated by Mec1/Rad53/Dun1-dependent phosphorylation after DNA damage or replication stress, which serves to prevent Crt1 from recruiting the Ssn6-Tup1 repressor complex to target promoters (Huang et al., 1998). Prompted by our analysis of Crt1 transcriptional targets, we found that combined expression of *RNR2*, *RNR3* and *RNR4* can substantially bypass the HU sensitivity of the *ccr4*Δ *dun1*Δ double mutant. It has been recently reported that *RNR* gene induction after DNA damage is defective in cells disrupted for Ccr4-Not complex function (Mulder et al., 2005). The severe sensitivity of the *ccr4*Δ *dun1*Δ strain to low levels of HU suggests that Dun1 and Ccr4 converge to regulate Crt1 and promote *RNR* gene induction (see Fig. 8 for proposed model of Crt1 regulation).

The mechanism whereby Ccr4 limits Crt1 activity appears multifarious. As one might have predicted, *CRT1* mRNA has a longer poly(A) tail in a *ccr4*Δ strain than in a wild-type strain. The known consequences of longer poly(A) tails are an increase in translation rate (Colgan and Manley, 1997; Preiss et al., 1998) and often an increase in mRNA stability (Tucker et al., 2001). In *ccr4*Δ strains, however, we found that *CRT1* mRNA is actually decreased to approximately 25-50% of wild-type levels. However, we did not observe a corresponding decrease in Crt1 protein abundance in the *ccr4*Δ strain; indeed, under conditions of replication stress, Crt1 appeared to be subtly increased in both *ccr4*Δ and *ccr4*Δ *dun1*Δ strains. An increased translation rate of the *CRT1* mRNA may underlie these effects; this notion is supported by isolation of *pbp1*Δ as a strong suppressor of *ccr4*Δ HU sensitivity. We note that *CRT1* mRNA is normally of very low abundance in wild-type cells (Huang et al., 1998) (this study), which may be linked to its unusual sensitivity to poly(A) tail length. Regardless of the precise mechanism that hyperactivates *CRT1* in the absence of *CCR4*, it is clear that the Crt1 level critically affects checkpoint function, *RNR* regulation and survival of checkpoint mutants (Huang et al., 1998).

Since Ccr4 regulates the polyadenylation state of myriad mRNAs (Muhlrad and Parker, 2005), at present it is not clear whether Ccr4 and poly(A) metabolism play a specific regulatory role – opposed to a passive role – in balancing various gene activities, including that of *CRT1*. It is possible that Ccr4 activity itself is regulated by replication stress either directly via post-translational modification or indirectly through the modulation of certain mRNAs species by RNA-binding proteins. The fact that *CHK1* and *CCR4* may act in the same pathway might argue for a specific regulatory function. Identification of crucial *cis*-elements on *CRT1* mRNA that regulate its translation or stability will help address its role during replication stress.

In fission yeast, overexpression of the Cid13 protein, a cytoplasmic poly(A) polymerase, rescues the HU sensitivity of many checkpoint mutants and, conversely, its deletion results in synergistic HU sensitivity (Saitoh et al., 2002). These phenotypes are also consistent with the notion that cytoplasmic poly(A) tail elongation of one or more crucial target genes results in replication-checkpoint defects. Although *suc22* (the *Schizosaccharomyces pombe* equivalent of *RNR2*) was identified as a candidate target for Cid13, it is unclear whether the shorter poly(A) tail of the *suc22* mRNA in *cid13*Δ mutant is specific for this transcript or whether it is the result of a more global response to the *cid13*Δ mutation. All told, these observations suggest a conserved link between mRNA metabolism and replication-stress tolerance in eukaryotes.

In addition to the requirement for proper poly(A) metabolism, our results also point to a connection between transcription and replication stress. Our insertional mutagenesis screen uncovered two partial loss-of-function

alleles of *MOT1*, which encodes an essential ATP-dependent transcription factor that regulates binding of Spt15 (TBP) to TATA boxes in target promoters (Adamkewicz et al., 2001). A recent proteome-wide approach identified Mot1 as a physical interacting partner of Crt1 (Gavin et al., 2002). Of particular interest, Ccr4-associated Not gene products have previously been functionally linked to Mot1 in the regulation of transcription (Collart, 1996). The isolation of *mot1* alleles that suppress the HU sensitivity of the *ccr4*Δ strain provides additional evidence that transcriptional defects compromise the ability to cope with replication stress (Mulder et al., 2005). Interestingly, Ccr4 is implicated in transcriptional initiation through genetic and physical interactions with the Paf1–RNA-polymerase-II complex and a *paf1*Δ strain is also sensitive to HU (Chang et al., 1999; Betz et al., 2002). Defective transcriptional activation at Crt1-repressed target promoters, through either ineffective transcriptional initiation or impaired chromatin remodelling, may contribute to the HU sensitivity of the *ccr4*Δ strain.

While evidently complex to interpret, our results emphasize the inter-connected nature of the network that controls the response to replication stress (see supplementary material Fig. S2 for a diagram of all known network interactions). The DNA damage response relies on appropriately controlled checkpoint signal transduction, DNA repair, DNA replication, oxidative metabolism, transcription, mRNA metabolism and cell cycle control, all of which must be balanced against one another to ensure optimal genome stability. That non-obvious and often subtle changes can perturb crucial cellular responses (Davierwala et al., 2005) is epitomized by the unanticipated connections between mRNA metabolism and the response to replication stress. Determining just how Crt1 and Ccr4 establish an appropriately poised transcriptional and post-transcriptional program to enable tolerance of replication stress will require further systems level interrogation of the response.

Materials and Methods

Plasmids and yeast strains

Standard methods were used for yeast culture and genetics (Amberg, 2005). All strains were congenic with BY4741 (S288c *MAT***a**) (Brachmann et al., 1998), except where noted as congenic with W303 *MAT***a**, and are listed in supplementary material Table S3. All plasmids are listed below. Mutant *CCR4* alleles were generated with the QuikChange system (Invitrogen) and cloned into *EcoRI* and *XhoI* sites of pRS416. The plasmids pGAP-RNR3 (pBAD079, *TRP1* CEN GAP-*RNR3*); pGAP (BAD054, *TRP1* CEN, empty vector); pGAP-RNR1 (pBAD070, *TRP1* CEN GAP-*RNR1*); pGAL (pDL054, *URA3* CEN); pGAL-RNR4 (pDL57, *URA3* CEN *GAL1-RNR4*) were provided by Steve Elledge (Harvard University). pGAL-FLAG (MT3164, *LEU2* CEN) and pGAL-RNR2-FLAG (MT3963, *LEU2* CEN *GAL1-RNR2-FLAG*) were generated with the Gateway (Invitrogen) vector recombination system (Ho et al., 2002). Integrations of *GAL1* promoters at genomic loci, and specific gene replacement with *Schizosaccharomyces pombe his5*+MX knockout cassettes were performed as described previously with the pFA6 family of plasmids (Longtine et al., 1998).

Systematic screen for HU sensitive deletion mutants

The haploid non-essential yeast deletion set (4812 strains) was arrayed by robotic pinning of colonies onto XY (YEPD + 100 mg/L adenine + 200 mg/L tryptophan) + 2% glucose medium containing 100 mM HU. Colonies were grown for one day at 30°C and subsequently re-pinned to XY-GLU+100 mM HU for another day of growth. Viability was assessed by visual comparison of colony size between exposure to drug and no drug and a relative score was assigned to arrayed colonies. All potential HU-sensitive strains were confirmed by streaking on XY-GLU or XY-GLU+100 mM HU to assess growth. HU sensitivity was confirmed using a spot test of serially-diluted cells on 100 mM HU (see below).

HU sensitivity assays

For assays on solid media, 5 μl of tenfold serially diluted cultures (starting OD_{600} of 1.0) were spotted on XY-plates containing either 2% glucose (GLU) or 2% galactose (GAL), in the presence or absence of indicated concentrations of HU. For liquid assays, cells were grown to an OD_{600} of 0.2 and HU was added to a final concentration of 200 mM. For determinations of colony-forming units (CFU), aliquots were removed from cultures at indicated intervals and spotted on XY-GLU plates with a Whitley Automatic Spiral Plater (WASP). Cells were grown at 30°C and CFU determined with WASP counting tables.

Microarrays and real time PCR

Cultures were grown in XY-2% glucose to an OD_{600} of 0.3 and total RNA was prepared by glass-bead lysis in phenol (Tyers et al., 1993). 50 μg total RNA was reverse transcribed to cDNA with SuperScript II (Invitrogen) using a poly(dT) oligonucleotid and directly labelled with either dCTP conjugated to Cy3 or Cy5. Differentially labelled experimental and control cDNA preparation were pooled, heated to 65°C for 2 minutes, then cooled to 50°C. Hybridization to oligonucleotide microarray was performed overnight at 37°C in 20:1:1 DIG-Easy Hybe (Roche):yeast tRNA(10 mg/ml):ssDNA (10 mg/ml). Arrays were rinsed in 1×SSC 0.1% SDS at 50°C followed by two washes of 15 minutes in 1×SSC 0.1% SDS. Arrays were rinsed three times in 0.1×SSC to remove SDS and gently spun dry. Scanning was performed with a GenePix 4000B (Axon Instruments). Spots were identified and quantitated with Quantarray 1.0 software (GSI Lumonics). Expression ratios were normalized and centred on the median by Quantarray Data Handler 3.0, and analyzed with AFM 4.0 (Breitkreutz et al., 2001). For real time PCR analysis, 50 μg total RNA was reverse transcribed and 1/50 of the reverse transcriptase (RTase) reaction was serially diluted twofold for analysis of *ACT1* and *CRT1* levels with a Taqman 7300 Real Time PCR system (Applied Biosystems) and standard conditions, using the following primers for *ACT1*: FOR: 5′-TGGA-TTCCGGTGATGGTGTT-3′; REV: 5′-TCAAAATGGCGTGAGGTAGAGA-3′ *ACT1* probe: TET fluorophore 5′-CTCACGTCGTTCCAATTTACGCTGGTTT-3′ For *CRT1*: FOR: 5′-CCGCCAGCATCACACACTTA-3′; REV: 5′-ACGATGA-TTTGCTCGCTATGG-3′ *CRT1* probe: FAM fluorophore 5′-TTGCCCCCCATGT-CAGTGAATATCCC-3′.

Protein detection

For immunoblot anaysis, 10 ml of culture was grown in XY-2% GLU to an OD_{600} of 0.3, centrifuged, and the pellet was frozen. Indicated samples were treated with 100 mM HU for 90 minutes. Cell pellets were lysed with glass beads in 20% TCA (Fischer). Extracts were resolved by 8% SDS-PAGE and transferred to nitrocellulose, followed by blocking with 5% skimmed milk in TBS-0.05% Tween (TBST). Crt1 protein levels were detected with a rabbit polyclonal antibody raised against the N-terminus of Crt1, provided by Joseph Reese (Zhang and Reese, 2005), and donkey anti-rabbit IgG-HRP secondary antibody. Control Pgk1 protein levels were detected with mouse monoclonal anti-yeast Pgk1 IgG (Molecular Probes) and sheep anti-mouse IgG-HRP. Rad53 (DAB001) was detected with an affinity-purified polyclonal rabbit anti-Rad53 antibody and goat anti-rabbit-HRP secondary antibody (Kanellis et al., 2003).

Rad53 phosphorylation and kinase assays

Rad53 kinase activity was determined with in situ kinase assay by [γ-^{32}P]ATP incorporation as described (Pellicioli et al., 1999). Briefly, cultures were treated with indicated concentrations of HU and lysed with glass beads in 20% TCA. Extracts were resolved by 8% SDS-PAGE and transferred to PVDF membrane. Proteins on the membranes were denatured with guanidine hydrochloride and re-natured overnight at 4°C with gentle shaking. Membranes were incubated with [γ-^{32}P]ATP to detect Rad53 kinase activity in situ (Pellicioli et al., 1999).

FACS analysis

Asynchronous cultures were grown to an OD_{600} of 0.2. Synchronization in early S-phase was achieved by treatment of mid-log phase cultures with 200 mM HU for 3 hours. HU was removed by washing cell pellets twice with fresh media lacking drug. Cell were pelleted, re-suspended in 70% ethanol and processed for FACS analysis using the Sytox Green nucleic acid stain (Invitrogen) exactly as described in Kanellis et al. (Kanellis et al., 2003). Analysis was performed on a Becton-Dickinson FACScalibur flow cytometer.

Transposon insertion suppressor screen

Transposon insertion mutagenesis was carried out as described (Ross-Macdonald et al., 1997). Briefly, a *ccr4*Δ::KANMX *MAT***a** strain was transformed with 1 μg of *Not*I-digested transposon Tn3-*LEU2* genomic library. After selection on SC minus leucine+100 mM HU for 3-5 days, putative suppressors were re-confirmed on 100 mM HU. Transposon insertions were isolated by *Rsa*I digestion of genomic DNA followed by re-ligation. PCR was performed on ligated DNA to amplify DNA adjacent to transposon-specific sequences, followed by sequencing (Ross-Macdonald et al., 1997).

Poly(A) tail length assays

Ligase-mediated poly(A) tests (LM-PAT) were performed essentially as described (Salles et al., 1999). All strains were grown in rich media supplemented with

adenine. Total RNA was isolated by the hot-phenol method and incubated with an excess of oligo(dT) primer, followed by addition of a linker oligo(dT-12) primer (5′-GCGAGCTCCGCGGCCGCGTTTTTTTTTTTT-3′) and T4 DNA ligase (Salles et al., 1999). PCR was performed as described by Salles et al. with primers designed within 100 bp upstream of the ORF stop codon. The following gene-specific primers were used for analysis: *CRT1-PAT*: 3′-GTCATGAAATTCGTCAATGGCC-3′; *RNR2-PAT*: 5′-GCCGGTGCTTTCACCTTCAACG-3′; *PGK1-PAT*: 5′-GATCTC-CCATGTCTCTACTGGTGG-3′; *ACT1-PAT*: 5′-CCGCTTTGGCTCCATCTTCC-ATG-3′. PCR products were separated on 2% high-resolution agarose (Invitrogen) and visualized with ethidium-bromide staining followed by laser scanning (Fuji FLA-5100). Poly(A) tail lengths were calculated on the basis of a 100 bp ladder (NEB) using FUJI MultiGauge software. Sizes were determined from peak intensities.

We thank David Lee, Steve Elledge, Marco Foiani, Rodney Rothstein, Michael Snyder and Joseph Reese for generous gifts of strains, plasmids, libraries and antibodies, Howard Lipshitz for helpful discussions, and Danielle Dewar and Marc Angeli for technical assistance. This research was supported by grants from the Canadian Institutes of Health Research (M.T.) and the National Cancer Institute of Canada with funds from the Canadian Cancer Society (D.D.). T.P. acknowledges support from the Australian Research Council and the Sylvia and Charles Viertel Charitable Foundation. M.T. holds a Canada Research Chair (CRC) in Bioinformatics and Functional Genomics; D.D. holds a CRC in Proteomics, Bioinformatics and Functional Genomics; R.N.W. holds a Terry Fox Foundation/National Cancer Institute of Canada Doctoral Studentship (Biomedical); M.A.C. holds a Canada Graduate Scholarship (Doctoral) from the Natural Sciences and Engineering Research Council of Canada; T.H.B. holds an NH and MRC Howard Florey Centenary Fellowship.

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