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Autorepression of Rfx1 Gene Expression: Functional Conservation from Yeast to Humans in Response to DNA Replication Arrest

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The yeast *Saccharomyces cerevisiae* Crt1 transcription repressor is an effector of the DNA damage and replication checkpoint pathway. Crt1 binds and represses genes encoding ribonucleotide reductase (RNR) and its own promoter, establishing a negative-feedback pathway. The role of Rfx1, the mammalian Crt1 homologue, remained uncertain. In this study we investigated the possibility that Rfx1 plays a similar function in animal cells. We show here that, like Crt1, Rfx1 binds and represses its own promoter. Furthermore, Rfx1 binding to its promoter is reduced upon induction of a DNA replication block by hydroxyurea, which led to a release of repression. Significantly, like Crt1, Rfx1 binds and represses the RNR-R2 gene. Upon blocking replication and UV treatment, expression of both Rfx1 and RNR-R2 is induced; however, unlike the results seen with the RNR-R2 gene, the derepression of the RFX1 gene is only partially blocked by inhibiting Chk1, the DNA checkpoint kinase. This report provides evidence for a common mechanism for Crt1 and Rfx1 expression and for the conservation of their mode of action in response to a DNA replication block. We suggest that Rfx1 plays a role in the DNA damage response by down-regulating a subset of genes whose expression is increased in response to replication blocking and UV-induced DNA damage.

Cells respond to DNA damage and replication blocking by attenuating cell cycle progression via transcriptional and posttranscriptional regulation of the components of the DNA replication, repair, and recombination pathways. Hydroxyurea (HU) specifically blocks DNA synthesis by inhibiting ribonucleotide reductase (RNR) (13). In *Saccharomyces cerevisiae*, RNR inhibition decreases deoxynucleoside triphosphate (dNTP) levels and activates a DNA checkpoint pathway composed of the Mec1, Rad53, and Dun1 protein kinases (21). Activation of the DNA checkpoint kinases stabilizes stalled replication forks, inhibits late-origin firing, blocks mitosis, and leads to activation of the RNR genes.

In *S. cerevisiae*, the product of the CRT1 gene is the downstream target of HU-activated signaling (21). Crt1 is a transcription repressor that recruits the general repressors Ssn6 and Tup1 to the promoters of several RNR genes. In response to a DNA replication block by HU, Mec1 (Atr) is activated to phosphorylate Rad53 (Chk1), which in turn phosphorylates the Dun1 kinase. Crt1 is phosphorylated in a Dun1-dependent manner, and the hyperphosphorylated form of Crt1 loses the capacity to bind DNA. Given the fact that Crt1 is a transcription repressor, this process leads to transcriptional activation of the target genes. Interestingly, Crt1 binds and represses its own promoter as well, generating a negative autoregulatory loop. It has been proposed that inhibition of an autoregulatory repressor in response to DNA damage is a strategy conserved throughout prokaryotic and eukaryotic evolution (21), but whether a similar Crt1 related mechanism exists in animal cells was not reported.

Crt1 shares significant sequence similarity with the DNA-binding domain of the mammalian Rfx proteins. Crt1 recognizes DNA elements closely resembling the mammalian X-box motif, a motif recognized by the Rfx transcription factors (15, 45). Rfx1 is a member of a family of proteins that is characterized by a unique winged helix DNA-binding domain, which is highly conserved in eukaryotic organisms (14, 17). The known Rfx family includes one member each in *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Caenorhabditis elegans*, and the fungus *Acremonium chrysogenum*, two members in *Drosophila melanogaster*, and five members in mammals (12, 14, 32, 38, 39, 43).

Members of the Rfx family of proteins are conserved throughout evolution and play diverse cellular functions. Several recent publications have identified specific functions for mammalian Rfx2 (42), Rfx3 (6), and Rfx4 (4, 5, 30). These functions are tissue and/or developmental stage specific, and at least some of them were previously described in regard to Rfx homologues in other organisms (12, 39). In contrast, Rfx1 is ubiquitously expressed, making it the best Crt1 orthologue candidate. Furthermore, like Crt1, Rfx1 has a transcription repression activity (22) and represses expression of genes such as PCNA and c-*myc* (26, 27, 46). Notably, Rfx1 possesses positive transcription activity as well (19). It has been suggested that the function of Rfx1 in supporting or repressing transcription depends on the promoter context (22).

The sequence and functional conservation between mammalian and yeast Rfx proteins is at the DNA-binding domain and the protein-protein interaction domain, as has been demonstrated by domain-swapping experiments (25). The level of conservation raises the possibility that the Rfx family has also retained its roles along the course of evolution. Such a model is supported by the recent identification of *Drosophila* Rfx2, dRfx2, that is involved in cell cycle regulation (32) in addition to the previously described dRfx that is involved in cilium formation similar to that seen with *C. elegans* DAF-19 and the mammalian Rfx3 (6, 12, 39).

In this report we demonstrate a functional conservation between human Rfx1 and the *S. cerevisiae* Rfx homologue Crt1 in repressing the activity of their own promoters, a repression

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that is relieved in response to hydroxyurea treatment. Our data implicate Rfx1 in cell cycle and DNA damage regulation and provide evidence that the negative regulatory loop is a universal and highly conserved mechanism in the cellular response to DNA damage.

MATERIALS AND METHODS

Plasmid construction. The Rfx1 promoter was produced by PCR using genomic DNA from HepG2 human hepatoma cells and Extensor Hi-Fidelity PCR Master Mix (ABgene). Primer sequences for production of the promoter were 5′AAACTCGAGGATGGACATTCATACTG and 5′TTTAAGCTTTGCGGAAACGCTTTTCG. The PCR product was cloned into the XhoI and HindIII sites of pGL3-basic vector (Promega). The HARfx1 constructs were previously described (22). The Rfx1 silent mutant was produced by PCR-mediated site-directed mutagenesis using *Pwo* DNA polymerase (Roche). The Ef1a-renilla vector used as internal control for luciferase assays was a generous gift from Yoram Groner.

For production of small interfering RNA (siRNA)-expressing plasmids, oligonucleotides 5′GATCCCCGATGGAAGGCATGACCAACTTCAAGAGAGTTGGTCATGCCTTCCATCTTTTTGGAAA and 5′AGCTTTTCCAAAAAGATGGAAGGCATGACCAACTCTCTTGAAGTTGGTCATGCCTTCCATCGGG were synthesized and cloned into pSUPER (8) (underlining indicates constant sequences inserted in all pSUPER primers). This plasmid produces siRNA targeted against nucleotides 1728 to 1746 on the Rfx1 mRNA, corresponding to amino acids 545 to 550 in the Rfx1 protein.

Cell culture and transfection. All cells were grown in Dulbecco's modified Eagle medium (Gibco) containing 100 U/ml penicillin and 100 μg/ml streptomycin, supplemented with 9% fetal calf serum. For experiments with HU, cells were passed three times in Dulbecco's modified Eagle medium with 9% charcoal-filtered serum before addition of 1 mM HU (Sigma) for 24 h. Amounts of 3 mM caffeine (Sigma) and 1 μM UCN-01 (drug synthesis and chemistry branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute) were added 30 min before the addition of HU. Transfections were done by the calcium phosphate precipitation method. Luciferase activity was measured using the Lucy3 luminometer (Anthos). UV irradiation was performed using a SPECTROLINKER XL-1500 UV cross-linker (Spectronics Corporation) in uncovered six-well plates (NUNC) without culture medium. pSUPER constructs for ATR, Chk1, and Chk2 were the generous gift of Reuven Agami. For knockdown of ATM, ATR, Chk1, and Chk2, MCF-7 cells were infected with retroviruses containing pRetroSUPER constructs (7) expressing the appropriate siRNAs. Stably transfected cells were selected with 10 μg/ml Puromycin.

Electrophoretic mobility shift assay (EMSA) and Western blotting. The gel shift assay was performed as previously described (24), with the slight modification that the extraction buffer did not contain Triton X-100 and the cells were lysed with five freeze-thaw cycles. The following oligonucleotides were used as probes: EP probe (5′-GATCTAGGCCGTTGCCGAGCAACG and 3′-ATCCGGCAACGGCTCGTTGCCTAG), x-box probe (5′-GATCCTTCCCCTAGCAACAGATA and 3′-GAAGGGGATCGTTGTCTATCTAG), Rfx1 upstream (pro1) probe (5′-TGGGTAGCAACAGTTGCCCCGGTGAGGG and 3′-ATCGTTGTCAACGGGGCCACTCCCTTTGV), Rfx1 downstream (pro2) probe (5′-AGGAAGCAACCCGGCAACGCGAGTCAACA and 3′-TCGTTGGGCCGTTGCGCTCAGTTGTTGTTG), and RNR-R2 pro probe (5′-AGGGTCGCAGCAACGCTCCCCCGCA and 3′-AGCGTCGTTGCGAGGGGGCGTGGGT).

The antibodies used for Western blot analysis included anti-Rfx1 polyclonal antibody produced in our laboratory, antihemagglutinin (anti-HA) monoclonal antibody (Babco), and anti-β-tubulin monoclonal antibody (Sigma). The blots were then reacted with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse antibody (Jackson) and developed using SuperSignal West Pico chemiluminescent substrate (PIERCE).

ChIP. Chromatin immunoprecipitation (ChIP) was performed according to the protocol of Ainbinder et al. (3). Briefly, formaldehyde cross-linked protein-DNA complexes were precipitated by incubation overnight with anti-Rfx1 polyclonal antibody or with rabbit preimmune serum as a negative control. The extract was then cleared by centrifugation and incubated for an additional 4 h with protein A/G-conjugated agarose beads (Santa Cruz). Precipitated DNA fragments were extracted and amplified with specific primers. The sequences of the primers used were 5′TCGGAACTAATAGTTTAGC and 5′CGCTTTTCGGAGGTCTCGG for the Rfx1 promoter or 5′CATTTTACTCACGGGGAC and 5′ACCGTTTAGGATTGCGTG for the RNR-R2 promoter. The GAPDH promoter was amplified using the primers described by Zhou et al. (48).

RNA analysis. Total RNA was extracted with TRI-Reagent (Molecular Research Center Inc.) according to the manufacturer's protocol. First-strand synthesis was performed using a Reverse iT 1st Strand kit (ABgene). Ten percent of the RT product and ReddyMix PCR master mix (ABgene) were used for PCR amplification of the specific fragment. Primer set 19743877a2 and 4557845a2 from the primer bank web site (http://pga.mgh.harvard.edu/primerbank/index.html) was used for the amplification of endogenous Rfx1 and RNR-R2, respectively. A primer corresponding to the sequence of the HA tag was used for the amplification of the transfected HARfx1 ΔN together with the antisense Rfx1-specific primer. Primers for β-actin were used as a control.

Primer sequences. The primer sequences were as follows: for Rfx1 sense, CTCCATGCCCATGTACGTGTC; for Rfx1 antisense, GGTGTGAGAGTAAGACTGGCTG; for HA sense, TGGCTTACCCATACGATGTTC; for RNR-R2 sense, AGGCTTCCTTTTGGACCGC; for RNR-R2 antisense, TTCTTGGCTAAATCGCTCCAC; for actin sense, ACCGCGAGAAGATGACCCAG; for actin antisense, CCATCTCGTTCTCGAAGTCCA.

RESULTS

Rfx1 binds its own promoter. Crt1, the yeast *Saccharomyces cerevisiae* Rfx homologue, binds and represses its own promoter (21). The consensus Rfx binding site identified in viral enhancer sequences is a palindrome consisting of two boxes in opposite orientation separated by a short spacer (10, 11). Unlike other transcription factors recognizing palindromic DNA sequences, Rfx1 is able to bind to a sequence consisting of only half of the palindrome; this ability is independent from its ability to dimerize (15, 34). To examine the possibility that mammalian Rfx1 binds its promoter as well, we used the MatInspector program from the GenomatixSuite software package to search for possible Rfx binding sites in the RFX1 upstream region. Two putative Rfx binding sites, each consisting of half of the palindrome, were identified in the RFX1 upstream region located 115 and 175 bp upstream to the transcription start site. These sites are fully conserved in mouse (Fig. 1A), as determined by alignment of the 2 kb upstream of the transcription start site of Rfx1 (overall identity of 52.4%) with the ClustalW algorithm (www.2.ebi.ac.uk/clustalw/). To investigate the regulation of the Rfx1 promoter we used the PromoterInspector program to identify the promoter sequence in the Rfx1 upstream region. We were able to identify a putative promoter sequence starting 355 bp upstream to the transcription start site. The program has also identified an additional putative promoter region located between 1,200 and 900 bp upstream of the Rfx1 transcription start site, with both Rfx binding sites located in the proximal element (Fig. 1A). To examine whether Rfx1 binds these sites corresponding oligonucleotides were used as probes for EMSA (Fig. 1B). Both the upstream (distal) and the downstream (proximal) sites formed a slow-migrating complex when incubated with cell extract (Fig. 1B, lanes 5 and 9). The patterns on both sites are identical to the one seen when using the known Rfx binding sequence from the major histocompatibility complex class II HLA-DR gene promoters (x-box) (35). The Rfx1 complexes were eliminated by competition with an excess of the unlabeled Rfx binding sequence from the hepatitis B virus enhancer (EP) (Fig. 1B, lanes 2, 6, and 10) but not with unrelated Hif1-responsive element sequence (Fig. 1B, lanes 3, 7, and 11), confirming the specificity of the complexes. Supershift experiments utilizing anti-Rfx1-specific antibodies confirmed the presence of Rfx1 in these complexes (Fig. 1B, lanes 4, 8, and

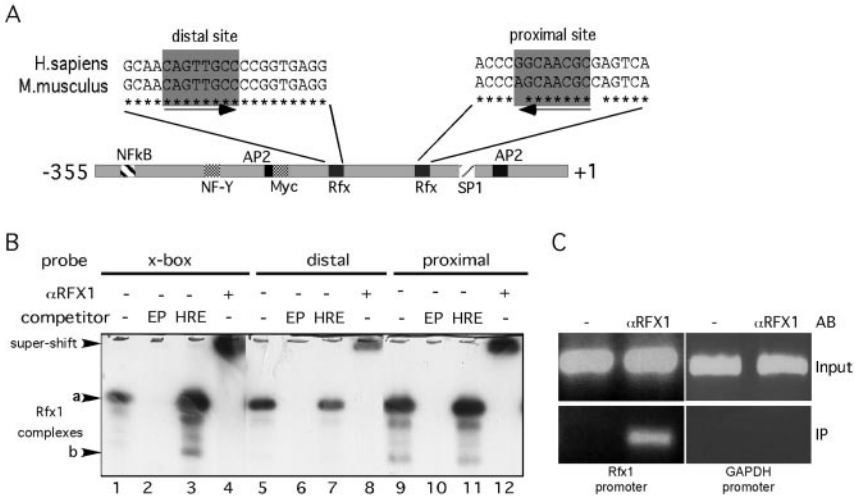

FIG. 1. Rfx1 binds to its promoter region: human Rfx1 binds to its own promoter region in vitro and in vivo. A. Alignment of the two putative Rfx binding sites identified in the human Rfx1 promoter (−355 to +1) with the equivalent site from the mouse Rfx1 promoter. Alignment was preformed using the ClustalW algorithm. B. EMSA with MCF-7 cell extract by use of different radiolabeled Rfx binding sites. The sequence used corresponds to the upstream site (distal; lanes 5 to 8) and the downstream site (proximal; lanes 9 to 12) found in the human Rfx1 promoter and the known x-box Rfx1 binding site of the major histocompatibility complex class II genes (lanes 1 to 4). The Rfx1 binding site from the hepatitis B virus enhancer (EP; lanes 2, 6, and 10) and the unrelated Hif1-responsive element (HRE; lanes 3, 7, and 11) were used as competitors. Anti-Rfx1-specific antibodies (αRfx1; lanes 4, 8, and 12) were used for Supershift experiments. C. Chromatin was immunoprecipitated from formaldehyde cross-linked MCF-7 cells with either preimmune rabbit serum (−) or anti-Rfx1 polyclonal antibodies (αRfx1). The presence of the Rfx1 and GAPDH promoter regions in the whole-cell extract (Input) and the precipitated complexes (IP) was determined by PCR.

12). These results suggest that Rfx1 binds the promoter of its gene at two sites.

To demonstrate that Rfx1 is able to bind its promoter in vivo we performed ChIP analysis by immunoprecipitating DNA cross-linked protein complexes from the MCF-7 cells either with specific anti-Rfx1 antibodies or with control antibodies. The region upstream of the Rfx1 gene was specifically precipitated with the anti-Rfx1 antibodies but not the control antibody (Fig. 1C, left panels), while the region containing the GAPDH transcription start site (48) was not precipitated by either antibody (Fig. 1C, right panels). Since the amplified sequence encompasses both of the binding sites we cannot determine whether Rfx1 binds both sites simultaneously. These data indicate that Rfx1 binds to its promoter in vivo as well as in vitro and is therefore a possible regulator of its own expression.

Rfx1 represses the activity of its own promoter. To investigate the regulation of the Rfx1 promoter the proximal promoter sequence and the 5′ untranslated region of Rfx1 were cloned upstream of the luciferase reporter gene (Fig. 2A). Luciferase expression was significantly increased (Fig. 2B), indicating that the cloned promoter sequence is functional. To investigate the effect of Rfx1 on the activity of its promoter we cotransfected the Rfx1 promoter reporter with a vector expressing the full-length Rfx1 into MCF-7 cells. Luciferase expression was reduced when Rfx1 was overexpressed in a dose-dependent manner (Fig. 2C), suggesting that human Rfx1 can repress its own promoter.

To verify that the repression of the Rfx1 promoter is mediated by the Rfx1 binding sites, we generated a series of mutants in which one or both sites are mutated (Fig. 2A). EMSA revealed that Rfx1 binding to the downstream site was completely abolished by the mutation (Fig. 2D, lanes 3 and 4), while the mutated upstream site still retains some residual

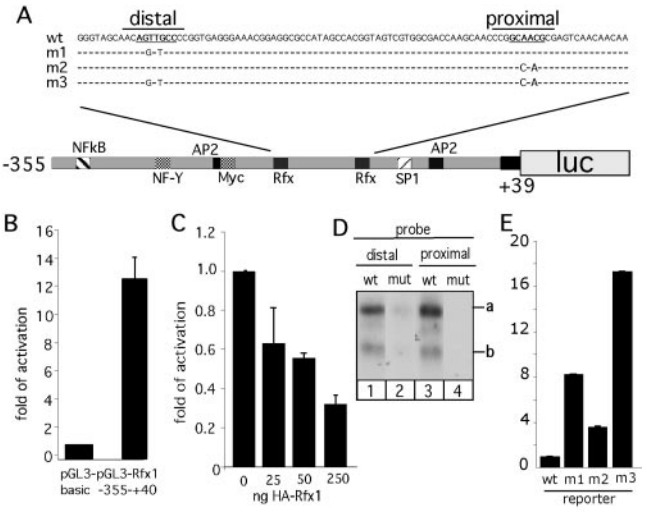

FIG. 2. Rfx1 represses the activity of its own promoter. A. Schematic drawing of the reporter construct containing the proximal Rfx1 promoter and the first exon of the Rfx1 mRNA (position −355 to +39) upstream of the luciferase reporter gene. Boxes mark Rfx and other transcription factor binding sites. Sequences of the constructs of Rfx1 promoter in which the distal (m1), proximal (m2), or both (m3) Rfx binding sites are mutated are shown on top. B. Luciferase activity from the Rfx1-luciferase construct compared to the activity from the pGL3-basic vector (Promega). C. Luciferase expression from the Rfx1 promoter in the presence of increasing amounts of pSG5-HARfx1. D. Oligonucleotides corresponding to the wt Rfx binding sites (wt; lanes 1 and 3) or the mutated sites (mut; lanes 2 and 4) were labeled and used as probes for EMSA with MCF-7 cell extract. The expected Rfx1 complexes a and b are indicated. E. Luciferase activity of the different reporter constructs. Reporter activity was normalized to an Ef1a-renilla internal control. Error bars represent standard deviations of at least three independent experiments done in duplicate.

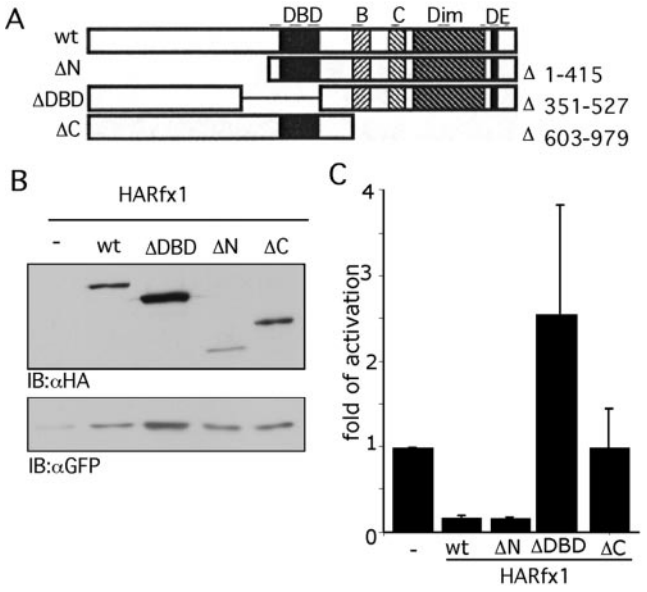

FIG. 3. Rfx1 transcription repression is mediated through its C terminus. A. Schematic drawing of HARfx1 and the deletion mutants used. All constructs include an HA tag at their N terminus. Boxes indicate the DNA-binding domain (DBD), conserved regions B and C, the dimerization domain (Dim), and the autoregulatory acidic region (DE). Deleted amino acids are indicated. B. Western blotting with anti-HA (Babco) of the different mutants transiently transfected in HEK293 cells and with anti-GFP to measure transfection efficiency. IB, immunoblot. C. Luciferase activity in MCF-7 cells transfected with Rfx1-luciferase reporter together with indicated different HARfx1 mutant constructs.

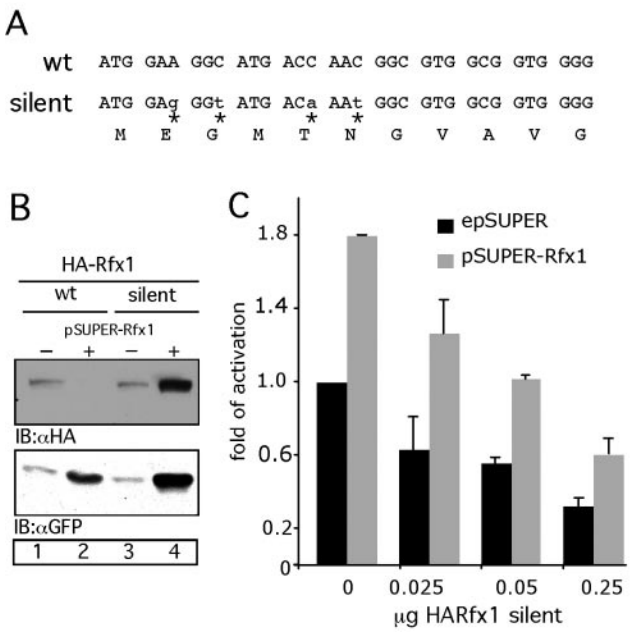

FIG. 4. The endogenous Rfx1 represses the Rfx1 promoter reporter. A. The sequence in Rfx1 targeted by pSUPER-Rfx1 (wt) was mutated so it would not be recognized. Mutated bases are in lowercase and marked with asterisks (silent). The mutations introduced no change in the protein sequence appearing at the bottom. B. Western blot of MCF-7 cells transfected with wt HARfx1 (wt; lanes 1 and 2) or with the silent mutant resistant to degradation by pSUPER-Rfx1 (silent; lanes 3 and 4) with (+) or without (−) pSUPER-Rfx1. pEGFP-C1 (Clontech) was used as internal control (lower panel). C. Luciferase activity of the Rfx1-luciferase reporter in the presence of pSUPER or pSUPER-Rfx1 and increasing amounts of pSG5-HARfx1 silent.

binding activity (Fig. 2D, lanes 1 and 2). Luciferase expression is higher from the mutant promoters than from the wild-type (wt) promoter, with the double mutant being more active than any of the single mutants (Fig. 2E). These results suggest that Rfx1 binding sites negatively regulate Rfx1 promoter activity in a cooperative fashion reminiscent of the findings in the yeast system, where the repression of Crt1p target genes is determined by the number and affinity of Crt1p binding sites (21).

Rfx1 transcription repression is mediated via its C terminus. Rfx1 has transcription activation and repression domains at its N and C termini, respectively (22). We used a series of Rfx1 deletion mutants, all of them nuclear proteins (23), to map the domain that mediates the repression of the Rfx1 promoter (Fig. 3A). These mutants are properly expressed, although to different levels, possibly due to their stability (Fig. 3B). The N-terminus deletion (ΔN) repressed Rfx1 promoter to the same extent as wt Rfx1 (Fig. 3C) even though its expression was lower, suggesting that deletion of the activation domain increases repression. The C-terminus-deleted Rfx1 (ΔC) lacking the repression domain (22) was inactive and unable to repress the Rfx1 promoter. Interestingly, an Rfx1 mutant lacking the DNA-binding domain (ΔDBD) behaved as a dominant-positive mutant and enhanced the activity of the Rfx1 promoter above the basal level. As this mutant is unable to activate transcription via binding to DNA we assume that it probably acts by sequestering a putative corepressor.

Rfx1 promoter activity is increased upon Rfx1 knockdown. In mammalian cells there are five members of the Rfx family with similar target DNA sequences (14). To show that the effect on the Rfx1 promoter is mediated by Rfx1 rather than by other members of the family we designed an siRNA expression vector to specifically knock down Rfx1 expression by use of pSUPER vector (8). Also, we designed an Rfx1 silent mutant that is expected to escape siRNA (Fig. 4A). This mutant is expressed at a level similar to the wt level when transfected into MCF-7 cells but is resistant to siRNA (Fig. 4B). In this experiment pEGFP-C1 was used as a control for the transfection. Notably, the siRNA-resistant Rfx1 mutant and green fluorescent protein (GFP) both show significantly higher expression in the presence of Rfx1 siRNA. The reason for this behavior is not clear, but the employed Rfx1- and GFP-expressing vectors use simian virus 40- and cytomegalovirus-based promoters, and each contains a putative Rfx binding site, which may mediate Rfx1-dependent repression. Transfection of MCF-7 cells with pSUPER-Rfx1 increased the activity of the Rfx1-luciferase reporter relative to that of the cells transfected with the control plasmid (epSUPER) (Fig. 4C), suggesting that the endogenous Rfx1 represses the transfected Rfx1 promoter. Under these conditions, repression of the Rfx1 promoter is resumed by expression of siRNA-resistant Rfx1 mutant. These data demonstrate that Rfx1 is a genuine repressor of its promoter.

Transfected Rfx1 represses the endogenous Rfx1 promoter. Next we performed experiments to repress the endogenous

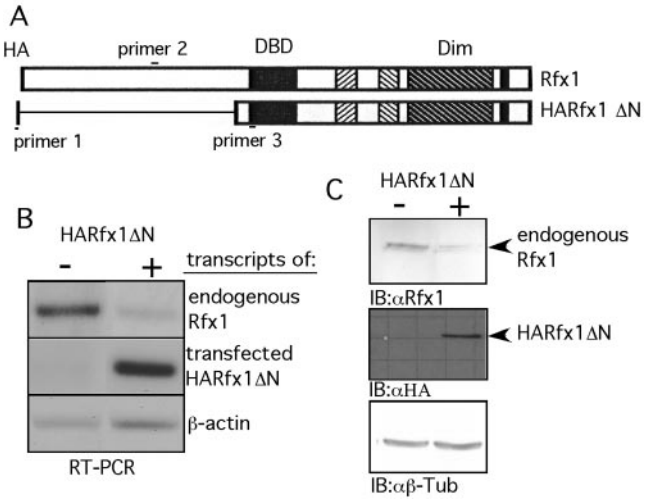

FIG. 5. Rfx1 represses the endogenous Rfx1 expression. Overexpression of HARfx1 ΔN represses the expression of endogenous Rfx1. A. Schematic drawing of Rfx1 indicating the location of PCR primers by numbers. Dim, dimerization domain. B. RT-PCR performed on RNA extracted from HEK293 cells transfected with HARfx1 ΔN (+) or with control empty pSG5 vector (−). The top panel shows the level of the endogenous Rfx1 RNA amplified with primers 2 and 3, while the middle panel shows the transfected HARfx1 ΔN amplified with primers 1 and 3. β-Actin was used as internal control (lower panel). C. Western blot on HEK293 cell extracts transfected with HARfx1 ΔN expression vector (middle panel). The level of the endogenous Rfx1 protein was determined using anti-Rfx1 antibodies (upper panel). β-Tubulin was used as a control (lower panel). IB, immunoblot.

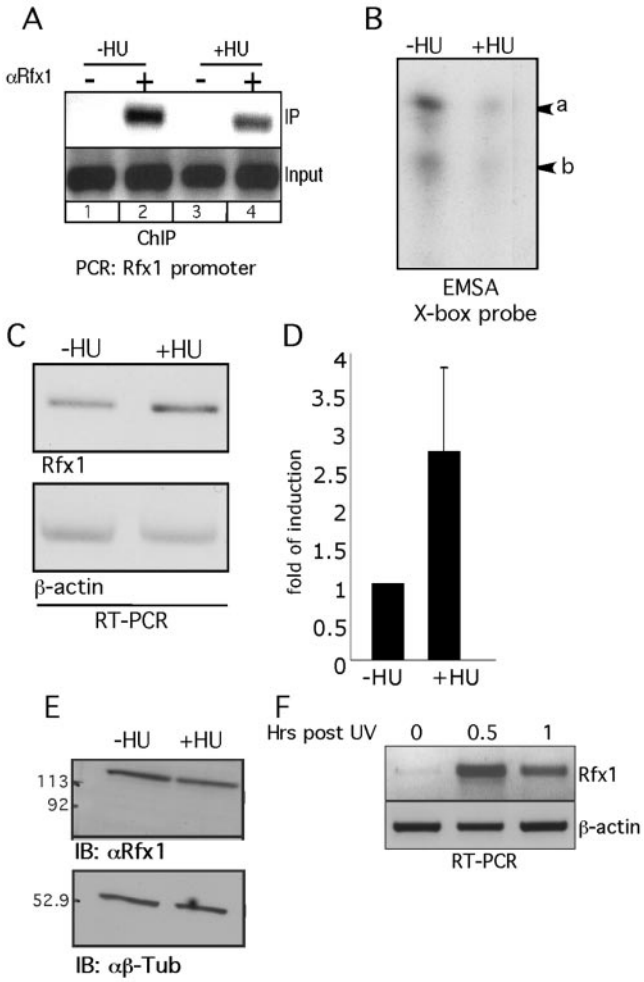

FIG. 6. HU reduces Rfx1 DNA binding. A. ChIP of the Rfx1 promoter. Cross-linked chromatin was precipitated with either anti-Rfx1 polyclonal antibodies (+; lanes 2 and 4) or with control preimmune rabbit serum (−; lanes 1 and 3) in the presence or absence of 1 mM HU. The amount of the Rfx1 promoter in the input and precipitated (IP) fractions was determined by PCR. B. EMSA with extract from MCF-7 cells grown in medium containing charcoal-filtered serum with or without 1 mM HU by use of the x-box sequence as probe. The expected complexes are marked a and b. C. RT-PCR on RNA extracted from MCF-7 cells treated as described for panel B using Rfx1-specific primers or β-actin-specific primers as controls. D. Quantification of Rfx1 RT-PCR band intensity by use of Exbam 3.0.4 software (Pixlock). Error bars represent three independent experiments. E. Western blot of the extracts described in for panel B. F. RT-PCR performed using total RNA from MCF-7 cells irradiated with 20 J/m^2 UV at different times postirradiation by use of Rfx1- and β-actin-specific primers.

Rfx1 promoter activity by Rfx1 overexpression. To this end we employed the HARfx1 ΔN mutant that is active in repressing the activity of the Rfx1 promoter (Fig. 3); at the same time, its level of expression can be easily distinguished from that of the endogenous Rfx1. Total RNA was extracted from cells transfected with HARfx1 ΔN and used as a template for reverse transcription-PCR (RT-PCR). Endogenous Rfx1 was detected using primers that specifically recognize the endogenous full-length Rfx1 sequence (Fig. 5A; primers 2 and 3). A second set of primers was used to specifically amplify the transfected ΔN construct (Fig. 5A; primers 1 and 3). The level of Rfx1 transcript is specifically reduced in cells expressing HARfx1 ΔN (Fig. 5B), supporting the idea of a role of Rfx1 in repressing the resident chromosomal Rfx1 promoter. Similar results were obtained by quantifying the level of the endogenous Rfx1 by use of anti-Rfx1 antibodies that do not recognize the transfected ΔN mutant (Fig. 5C). These data indicate that the resident chromosomal Rfx1 promoter undergoes repression by Rfx1, establishing an autorepression-regulatory loop.

Hydroxyurea increases Rfx1 expression by blocking its DNA binding activity. The DNA replication arrest checkpoint pathway regulates the DNA binding of the yeast Rfx homologue (21). The yeast Crt1 binding to its promoter is reduced upon treatment with HU, a known activator of the replication arrest checkpoint. To test whether the mammalian Rfx1 responds to this block we treated cells with HU and followed Rfx1 binding to its promoter. HU induces replication arrest by repressing RNR and thus depleting cellular dNTPs. To improve the effect of HU treatment, cells were fed with charcoal-filtered medium. Chromatin was immunoprecipitated from HU-treated or untreated cells with anti-Rfx1 antibodies, and the amount of the RFX1 DNA promoter in the precipitated fraction was determined by PCR (Fig. 6A). DNA binding of Rfx1 was analyzed in vitro by EMSA using the x-box probe (Fig. 6B). The level of Rfx1 binding was reduced after HU treatment in both experiments, indicating that Rfx1 is much less efficient in binding to its target sequences after HU treatment, in similarity to its yeast homologue. The level of the Rfx1 mRNA was determined by RT-PCR (Fig. 6C), and the intensity of the bands was

quantified using Exbam 3.0.4 software (Pixlock) (Fig. 6D). The levels of Rfx1 mRNA increased upon HU treatment, correlating a decreased level of binding with derepression of Rfx1. The level of protein under these conditions was not significantly changed (Fig. 6E), indicating that the regulation of the level of Rfx1 binding is mediated via modification of Rfx1 affinity to DNA rather than by changes in the protein level. However, due to the autorepression activity of Rfx1 a higher level of the Rfx1 protein was expected upon HU treatment, and the fact that this is not the case led us to conclude that some Rfx1 destabilization must occur. Next we analyzed the level of RFX1 mRNA under conditions of UV radiation that are expected to activate the same signaling pathway as HU (1). RFX1 RNA level was significantly induced within 30 min (Fig. 6F). These results suggest that Rfx1 autorepression is released upon activating the DNA replication block and UV-induced DNA damage signaling.

Rfx1 binding to the RNR-R2 promoter is reduced in response to hydroxyurea. The major targets of the yeast Rfx homologue are the different subunits of RNR (21). In mammals RNR consists of a constitutively expressed R1 subunit and a cell cycle-checkpoint-induced R2 subunit (16). We speculated that in similarity to that of yeast, the animal cell Rfx1 regulates the expression of RNR and binds specifically to the promoter of the R2 subunit. The promoter of the human R2 subunit has been characterized previously (33), and we could indeed identify a putative Rfx binding site, an x-box, at position −533. EMSA revealed that the putative x-box binds Rfx1 (Fig. 7A). Next we examined the association of Rfx1 with the RNR-R2 promoter in the cells by employing ChIP. The RNR-R2 promoter was specifically precipitated by anti-Rfx1 antibodies and not with preimmune serum (Fig. 7B). Remarkably, treatment with HU significantly reduced the binding of Rfx1 to the RNR-R2 promoter. To test whether in response to HU the release of Rfx1 from DNA is in correlation with an increase in transcription of the RFX1 and RNR-R2 genes we extracted RNA from HU-treated or untreated cells and examined the level of the Rfx1 and RNR-R2 mRNA by RT-PCR. The level of both transcripts was elevated in HU-treated cells (Fig. 7C). These data suggest that Rfx1 represses RFX1 and RNR-R2 expression and that, in response to DNA replication block stress, Rfx1 binding to the target promoters is reduced, with concomitant transcription activation.

Regulation of RFX1 expression by ATR-induced downstream signaling. The signaling pathway that is activated by HU is highly conserved and consists of the PI3K family kinase ATR and the effector kinase Chk1 (1). To test whether Rfx1 is a target of the ATR signaling pathway we have used caffeine, an inhibitor of ATR, and UCN-01, a specific inhibitor of Chk1 (37, 47). Caffeine prevented the accumulation of both Rfx1 and RNR-R2 mRNAs in response to HU treatment (Fig. 7C), indicating that Rfx1 is likely to be a target of the ATR signaling pathway. This hypothesis was tested by UV treatment, a known inducer of ATR. Under these conditions both Rfx1 transcription and, to a lower extent, R2 transcription are induced very rapidly (Fig. 8A). Caffeine treatment resulted in a higher basal level of Rfx1 RNA that is not further accumulated upon UV radiation. Also, the level of induction was less noticeable in the presence of Chk1 inhibitor. Interestingly, R2 transcription behaved in a similar manner. On the other hand, while UCN-01 had a significant effect on the accumulation of RNR-R2 mRNA in response to HU, it had only a minor effect on Rfx1 mRNA levels, suggesting that Rfx1 is partially regulated in a Chk1-independent manner (Fig. 7C).

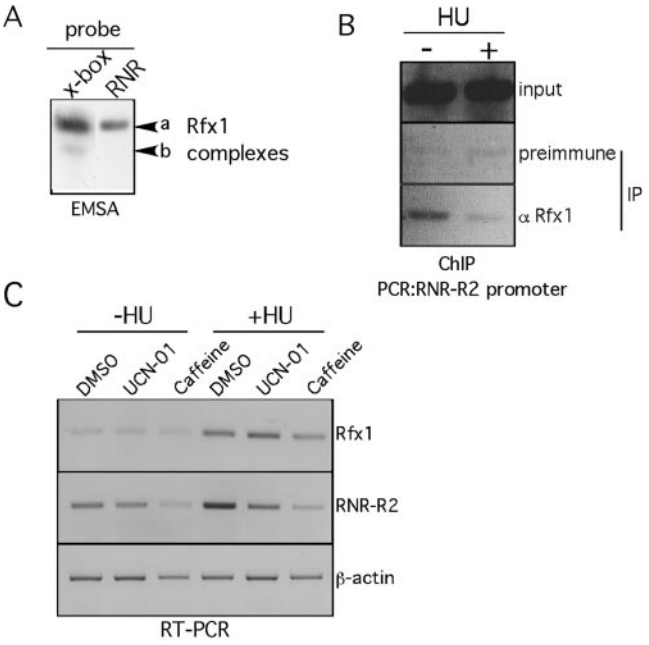

FIG. 7. Rfx1 binds to the RNR-R2 promoter and is regulated by HU. A. EMSA with MCF-7 extract by use of the x-box or the putative Rfx binding site from the RNR-R2 promoter as probe. B. ChIP with MCF-7 cells grown in medium containing charcoal-filtered serum in the presence or absence of 1 mM HU. Chromatin was precipitated using anti-Rfx1 antibodies (αRfx1; bottom panel) or control rabbit preimmune serum (middle panel). The level of the RNR-R2 promoter in the input and precipitated (IP) fractions was determined by PCR. C. RT-PCR of MCF-7 cells grown as described for B. As indicated, 3 mM caffeine or 1 μM UCN-01 was added 30 min before the addition of HU. Dimethyl sulfoxide was added as a control to all samples not containing UCN-01.

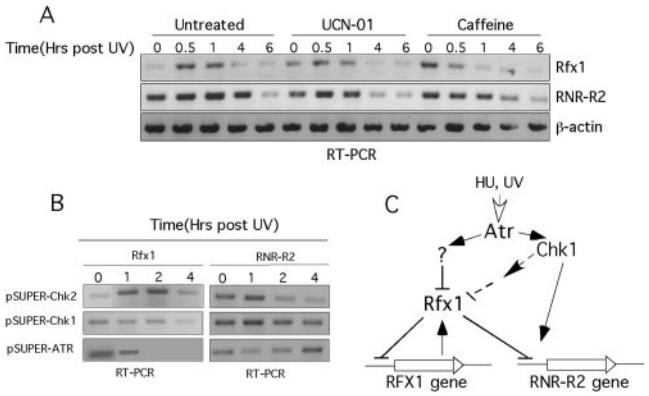

FIG. 8. RFX1 gene expression is UV responsive. A. RT-PCR using total RNA from UV-irradiated MCF-7 cells. UCN-01 (1 μM) or caffeine (3 mM) was added 30 min before irradiation (20 J/m^2). B. RT-PCR of MCF-7 stably expressing siRNA against Chk2, Chk1, or ATR irradiated with 20 J/m^2 UV. C. A suggested model for the interaction of Rfx1 with the other components of the DNA replication checkpoint pathway.

The ATR-Rfx1 signaling was also examined under conditions of UV radiation. Time course experiments clearly showed that the RFX1 gene and, to a lesser extent, the RNR-R2 gene are upregulated under UV treatment within 30 to 60 min (Fig. 8A). Upon inhibition of Chk1 by UCN-01 the response of both genes to UV was lower but not completely diminished. Here again, caffeine-treated cells showed a higher initial Rfx1 RNA level that was not further induced by UV but rather sharply reduced. The response of the RNR-R2 gene to UV was also diminished in the caffeine-treated cells. Similar results were obtained while analyzing UV response of Chk1 and Atr but not Chk2 knockdown cells, suggesting the specificity of the action of the drug inhibitors. These data suggest that Rfx1 is a downstream target of Atr that acts to modulate its own transcription and RNR-R2 transcription via a repressive mechanism (Fig. 8C).

DISCUSSION

The capacity of cells to properly respond to DNA damage and replication blocks has stimulated many studies that aimed at resolving the underlying molecular mechanisms. The principle of a checkpoint-signaling pathway has provided a satisfactory model to explain the molecular sequence of events that is triggered by these stresses. The evolutionary conservation of these processes is remarkable not only on the level of similarity between the components but also with respect to conservation of the actual mechanism. Taking advantage of this notion, we were able to show that the DNA binding Rfx1 protein is an important component of replication block signaling. Rfx1 represses its own promoter and the RNR-R2 promoter in a manner similar to that seen with the Crt1 yeast homologue.

The binding of Rfx1 to its promoter was demonstrated in vitro by employing EMSA and in the cells by performing ChIP assays. The fact that in the context of the Rfx1 promoter Rfx1 plays the role of repressor was demonstrated by either overexpressing Rfx1 or by knockdown experiments. We could further show that under Rfx1 knockdown conditions ectopic expression of an siRNA-resistant Rfx1 is sufficient to repress the Rfx1 promoter. Significantly, the endogenous Rfx1 gene expression can be repressed by utilizing an Rfx1 dominant-negative mutant. Altogether, these experiments indicate that Rfx1 is autorepressed. In this regard Rfx1 is surprisingly similar to the yeast homologue Crt1 not only on the level of structure (25) and target DNA sequence but also on the level of their transcription regulation.

The mechanism of transcription repression by Rfx1 is not clear yet. The yeast homologue represses transcription by recruiting the Gro/TLE-related Ssn6/Tup1 repressor complex (21). The human Rfx1 does not contain a known TLE binding motif, and human TLE1 was undetectable in Rfx1 immunoprecipitation (unpublished results). In the context of the PCNA gene the tumor suppressor p107 has been regarded as the modulator of Rfx1 (27). Notably, the activity of Rfx1 is context dependent, and certain promoters are subject to positive regulation by Rfx1 as well. Rfx1 contains both activation and repression domains that can neutralize one another (22) and can generate distinct DNA-protein complexes (24). Furthermore, Rfx1 is the target of several signaling pathways in addition to the DNA replication and UV-induced DNA damage pathways (2, 9, 29, 46). It is therefore possible that the mode of Rfx1 action is also determined by the nature of the incoming signals. Given the fact that upon HU treatment Rfx1 is no longer in association with both RFX1 and RNR-R2 promoters, the contribution of the positive Rfx1 domain in regulating transcription of these promoters is minimal if it exists at all.

Transcription repression appears to be an emerging strategy in regulation of genes whose expression correlates with the activation of replication block signaling. This strategy is reminiscent of the SOS response in *Escherichia coli* (reviewed in reference 40). Our study provides evidence that this strategy is conserved up to the level of animal cells. Furthermore, the fact that the promoters of both RFX1 and CRT1 genes contain multiple Rfx1 binding sites indicates conservation between the two genes at the level of promoter structure as well. Rfx1 binds its promoter at two sites, and deletion of each partially relieves the repression of the Rfx1 promoter, suggesting that the two sites are acting cooperatively. In the context of yeast it has been demonstrated that the timing and extent of derepression of Crt1 target genes are controlled by the number and strength of the x-boxes in their promoters (21). This may provide a reasonable explanation for why the overall structure of the promoter is conserved in evolution.

In both *S. cerevisiae* and *S. pombe* the single known Rfx homologue is important for regulation of cell cycle (21, 43). A recent discovery of an additional Rfx family member in *Drosophila* species that is also involved in cell cycle regulation (32) highlights the possibility that the Rfx family retained the function of its yeast ancestors in higher organisms, with additional family members taking on new roles. In humans the Rfx protein family consists of five members which function in various biological systems (6, 10, 12, 14, 38, 39, 42, 43). On the basis of domain-swapping experiments we have concluded that Rfx1 is the homologue of Crt1 (25). Also, unlike the other mammalian Rfx homologues that function in specific tissues and/or developmental stages, Rfx1 is ubiquitously expressed, supporting the possibility that like Crt1, Rfx1 has a general and basic cellular function. An Rfx1-like function might even exist in prokaryotes. For example, the DNA-binding domain of the origin binding protein of bacteriophage P4 shows a high degree of structural similarity to the DNA-binding domain of Rfx despite a very low level of sequence similarity (44). A bacterial Rfx homologue, therefore, might be identified in the future with an increase in the number of known protein structures.

Depletion of dNTPs leads to stalling of replication forks and activation of the replication checkpoint signaling pathway. The upstream components of the pathway are conserved between yeast and mammals (1, 36). The major effector kinase of the pathway in mammals is Chk1 (28). Chk1 is phosphorylated by ATR in response to various stress conditions such as replication arrest and UV-induced DNA damage, which leads to activation of Chk1 and phosphorylation of its downstream targets (20). Rfx1 contains a consensus Chk1 phosphorylation site (31) on serine 753. To test whether Rfx1 is a target of the ATR signaling pathway we have used caffeine, an inhibitor of ATR, and UCN-01, a specific inhibitor of Chk1 (37, 47). Caffeine prevented the accumulation of both Rfx1 and RNR-R2 mRNAs in response to HU or UV treatment, suggesting that Rfx1 is a likely target of the ATR signaling pathway. However, we could not detect any significant phosphorylation of Rfx1 by

Chk1 either in vitro or in cells that are infected with Rfx1 and Chk1 recombinant baculoviruses (data not shown). Consistent with this notion is the finding that the Chk1 inhibitor, while blocking the activation of RNR-R2, had only a minor effect on the HU- and UV-exerted Rfx1 transcription activation. Rfx1 contains about 20 S/TQ sequence motifs, the target sequence of phosphorylation by ATR. It is therefore possible that Rfx1 is a direct substrate of ATR. Notably, although in yeast Crt1 is the most downstream target of the Mec-1-Rad53-Dun1 cascade, the question of whether Crt1 is a direct substrate of one of these kinases, if any, remains unresolved (21).

In yeast the genes that encode the different subunits of RNR are under Crt1 repression (18, 21, 41). In mammals the RNR subunits are not coregulated. The R1 subunit is constitutively expressed, whereas the R2 subunit (RNR-R2) is a cell cycle-checkpoint-induced gene (16). An Rfx1 binding site was identified at the region of the RNR-R2 promoter and on the basis of EMSA and chromatin immunoprecipitation assays was found to be a functional binding site both in vitro and in vivo. We further show that the activity of the RNR-R2 promoter is derepressed upon HU treatment, in good correlation with a significant reduction in the binding of Rfx1 to the RNR-R2 promoter. These data suggest that RNR-R2 is under Rfx1 repression as well.

This study describes the signaling pathway in animal cells that is activated upon cellular stress, such as replication block and UV irradiation, to derepress the desired target genes (Fig. 8C). A remarkable feature of this signaling is its conservation from yeast to humans not only at the level of the constituents of the pathway but also at the level of the mechanism, as exemplified by the finding that the downstream target gene functions as a repressor and is subject to autorepression. Nevertheless it appears that the animal cells have acquired a certain level of additional complexity. Rfx1 regulates expression of PCNA and c-Myc (27, 46) and is expected to play an important role in cell proliferation. This is not the case with Crt1 (18, 45). In addition, Crt1 is not an essential gene, as demonstrated by the fact that Crt1 knockout yeast species grow properly under normal conditions (49). In contrast, Rfx1 knockdown gives rise to a block in cell proliferation (our unpublished data). This may suggest that along the course of evolution, a tighter linkage has been generated between DNA damage stress response and cell proliferation, possibly to eliminate growth of defective cells in multicellular organisms.

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