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Rtr1 Is the *Saccharomyces cerevisiae* Homolog of a Novel Family of RNA Polymerase II-Binding Proteins*

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Cells must rapidly sense and respond to a wide variety of potentially cytotoxic external stressors to survive in a constantly changing environment. In a search for novel genes required for stress tolerance in *Saccharomyces cerevisiae*, we identified the uncharacterized open reading frame YER139C as a gene required for growth at 37°C in the presence of the heat shock mimetic formamide. YER139C encodes the closest yeast homolog of the human RPAP2 protein, recently identified as a novel RNA polymerase II (RNAPII)-associated factor. Multiple lines of evidence support a role for this gene family in transcription, prompting us to rename YER139C *RTR1* (regulator of transcription). The core RNAPII subunits RPB5, RPB7, and RPB9 were isolated as potent high-copy-number suppressors of the *rtr1Δ* temperature-sensitive growth phenotype, and deletion of the nonessential subunits RPB4 and RPB9 hypersensitized cells to *RTR1* overexpression. Disruption of *RTR1* resulted in mycophenolic acid sensitivity and synthetic genetic interactions with a number of genes involved in multiple phases of transcription. Consistently, *rtr1Δ* cells are defective in inducible transcription from the *GAL1* promoter. Rtr1 constitutively shuttles between the cytoplasm and nucleus, where it physically associates with an active RNAPII transcriptional complex. Taken together, our data reveal a role for members of the *RTR1/RPAP2* family as regulators of core RNAPII function.

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the LEU2 and YDR066C were obtained from Open Biosystems (Huntsville, AL). An addition to media included dextrose (or another sugar, as indicated). Synthetic complete (SC) medium culture optical density at 600 nm of 1.0, and the resulting culture was transferred out by streaking or by serial dilution using 1/10 dilution steps with a starting (100

interactions. We have therefore named this gene YER139C. The cellular and biological significance of the genetic and biochemical interactions was demonstrated by translating the transcriptionally active form of Rpb1. Cells expressing Yer139c shuttles between the cytoplasm and nucleus and physically associates transcriptional regulators. We show that Yer139c expression through genetic interaction with RNAPII and with terization of this gene strongly links it to regulation of gene expression through genetic interaction with RNAPII and with associated transcriptional regulators. We show that Yer139c shuttles between the cytoplasm and nucleus and physically interacts with the transcriptionally active form of Rpb1. Cells lacking YER139C display a transcriptional defect in inducible expression from the GAL1 promoter, demonstrating the physiological significance of the genetic and biochemical interactions. We have therefore named this gene RTR1 (for “regulator of transcription”). A recent proteomic analysis has identified the human homolog of RTR1 among a group of novel RNAII-associated proteins, supporting our proposed in vivo role for Rtr1 in transcription (30).

MATERIALS AND METHODS

Sacharomyces cerevisiae methodology. S. cerevisiae strains were grown on media containing 2% dextrose, sucrose, or galactose as indicated. Unless otherwise noted, strains were grown at 30°C. Rich yeast extract-peptone-dextrose (YPD) growth medium was prepared containing 1% yeast extract, 2% peptone (or another sugar, as indicated). Synthetic complete (SC) medium lacking the appropriate nutrient for plasmid selection was purchased from Sun- rise Science Products (San Diego, CA). Other additions to media included formamidine (2%), 6-a-azauracil (6AU) (100 μg/mL), and mycophenolic acid (MPA) (100 μg/mL) (Sigma Aldrich, St. Louis, MO). Standard yeast propagation and transformation procedures were employed (31). Plate growth assays were carried out by streaking or by serial dilution using 1/10 dilution steps with a starting culture optical density at 600 nm of 0.1, and the resulting culture was transferred with a multipronged replicating tip (Sigma). Strains used in this study are listed in Table 1. Haploid strains carrying kanMX-marked disruptions in RTR1, RPB4, RPB9, GAL11, MEDI, SOHI, SRS5, CCR4, CDC73, DIST1, ELP2, SPT4, RPB4, and YDR066C were obtained from Open Biosystems (Huntsville, AL). An independent RTR1 disruption cassette was generated by PCR amplification of the LEU2 gene followed by cloning into pBluescript II. Then, 250-bp regions flanking the 5’ and 3’ sides of RTR1 were cloned upstream and downstream of the LEU2 gene, respectively. The entire construct was excised by restriction digest and transferred into target strains. Disruption of RTR1 by homologous recombination was selected by growth on media lacking leucine and confirmed by PCR. Sequences for all oligonucleotide primers used in this study are available upon request.

Plasmid construction. Unless otherwise noted, ORFs were amplified from BY4741 genomic DNA by use of standard PCR protocols and cloned into expression vectors by use of restriction endonuclease sites engineered into the amplifying oligonucleotide primers by use of standard DNA digestion and ligation protocols. The specific cloning sites and binding vectors are identified together with the list of plasmids in Table 2. Vent DNA polymerase, T4 ligase, and restriction endonucleases were purchased from New England Biolabs (Beverly, MA). To construct both green fluorescent protein (GFP)-tagged and protein A-tagged versions of Rtr1, the coding region of S. cerevisiae RTR1 (including 300 bp from the 3’ untranslated region) was amplified from R545h genomic DNA by use of PCR and cloned into GFP and protein A expression vectors described elsewhere (6). Constructs were verified by DNA sequencing. Expression of the N-terminally GFP- or protein A-tagged Rtr1 fusion proteins was analyzed by immunoblotting. A tripeptide hemagglutinin (HA)-tagged allele of RTR1 was constructed using a PCR-based approach. Oligonucleotides containing sequences flanking the RTR1 stop codon and containing additional homology to the template plasmid pFA6a-3HA-His3MX6 amplified a cassette used to modify p416CUP-RTR1 via homologous recombination in yeast cells. This construct was then used as a template for the transformation of the human erythroleukemic K562 cells remaining to express the Rtr1 C-terminal domain and histidine residues within the RTR1 cysteine-rich domain. This was accomplished using overlap extension PCR. Complementary primers were designed to mutate the cysteine at position 73 to serine and the cysteine and histidine residues at positions 112 and 116, respectively, to serine. These primers were used in PCRs with primers flanking the start or stop codons to generate two products with an approximately 20-nucleotide overlap in the respective 3’ and 5’ ends. These products were used as templates in a third PCR that included only ORF-flanking primers to reconstruct a full-length ORF containing the mutation(s) of interest. V5-epitope coding sequence was included in the oligonucleotides for construction of all V5-tagged constructs. All constructs were verified by sequencing. Protein extraction, affinity purification, and immunoblot analysis. Cells were harvested and resuspended in TEGN buffer (20 mM Tris [pH 7.9], 0.5 mM EDTA, 50 mM KCl, 150 mM NaCl) with protease inhibitors (sodium vanadate, 1 mg/mL; pepstatin A, 2 μg/mL; leupeptin, 1 μg/mL; phenylmethylsulfonyl fluoride, 1 mM; chymostatin, 2 μg/mL; Sigma) followed by the addition of acid-washed glass beads. The samples were then lysed by agitation using a microtube mixer (Tomy MT-360) for five rounds of 1.5 min of lysis in each row followed by 1.5 min on ice. The lysate was then cleared by centrifugation at 4,500 × g at 4°C for 7 min. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose (Bio-Rad, Hercules, CA) for further analysis. Blots were treated with a blocking solution (MPA) for 30 min followed by detection of protein A (tandem affinity purification [TAP]-)tagged proteins expressed in yeast were performed as described previously (4). 12CA5 monoclonal antibody recognizing the HA epitope was purchased from Roche Diagnostics (Indianapolis, IN) and used at a dilution of 1:5,000. V5 monoclonal antibody recognizing the V5 epitope was purchased from Invitrogen and used at a dilution of 1:500. Mouse monoclonal antibodies SWG16 (MMS-126R), H5 (MMS-129R), and H14 (MMS-134R), all recognizing RNAPII, were obtained from Covance (Princeton, NJ) (9, 51, 62). A slot-blot approach was used to react individual lanes with equivalent loads from the same blot with all three anti-RPB1 antibodies. High-copy-number suppressor screening. The rtr1 strain was transformed with a genomic high-copy-number library in the pYEP24 vector backbone (10). A total of 2,000 transformants grown at 37°C in the presence of 2% formaldehyde to select for suppressing colonies, and 23 were recovered. Plasmids responsible for suppression were rescued and placed into independent classes via restriction digestion with HindIII, resulting in three distinct groups. A representative member of each group was then sequenced using primers flanking the genomic fragment insertion site. Individual genes from each sequenced fragment were cloned into plasmid p423GPD and tested for suppression ability. After identification of RPB5 and RPB9 as high-copy-number suppressors, the remaining RNAPII subunits were also cloned into p423GPD as described above and examined for suppression ability. Overexpression plasmids containing RPB1 and RPB2 were kind gifts from Nancy Woychik (Robert Wood Johnson Medical School, New Brunswick, NJ).

Microscopy. Expression and localization of the GFP-Rtr1 fusion protein were done essentially as described previously (5). Specifically, the rtr1:kanMX4 deletion strain or yeast strains encoding the spo11-1 conditionally temperature-sensitive exportin mutant were transformed with a CEN plasmid encoding GFP-Rtr1. The cells were grown at 25°C to an optical density at 600 nm of 0.5 and incubated with 4.6-diamidino-2-phenylindole (DAPI) at a final concentration of 2.5 μg/mL for 45 min to stain nuclei. A fraction of the cell culture was shifted to the restrictive temperature of 37°C for 8 min and subsequently analyzed by fluorescence microscopy using a Nikon Eclipse TE2000-U fluorescence microscope equipped with an DS-5M (Nikon Instruments Europe B.V., Dusseldorf, Germany) and processed in Adobe Pho- toshop 4 (Adobe Systems Inc., San Jose, CA).

Northern blot analysis of gene expression. Expression of galactose-inducible and constitutively expressed genes was determined by Northern analysis as fol-
TABLE 2. Plasmids

| Plasmid       | Description (cloning sites)                  | Reference or source |
|---------------|----------------------------------------------|---------------------|
| p416CUP1      | Low copy number; weak constitutive promoter  | 36                  |
| p416CUP1-RTR1-HA | C-terminal HA-tag fusion (SpeI/XhoI)       | This study          |
| p416CUP1-RTR1::5H9004-HA | C-terminal HA-tag fusion (SpeI/XhoI) | This study          |
| p416CUP1-RTR1::5H11001-HA | C-terminal HA-tag fusion (SpeI/XhoI) | This study          |
| pFA6a-3HA-His3MX6 | Contains both a triple HA epitope and a hexahistidine coding sequence | 43                  |
| p416CUP1-GFP-RTR1 | C-terminal GFP fusion (SpeI/XhoI)           | This study          |
| p423GPD        | High copy number; strong constitutive promoter | 48                  |
| p423GPD-RTR1-V5 | C-terminal V5-tag fusion (SpeI/XhoI)        | This study          |
| p423GPD-V5-RTR1 | N-terminal V5-tag fusion (SpeI/XhoI)        | This study          |
| p423GPD-V5-N-RTR1 | N-terminal V5-tag fusion to N terminus of RTR1 (amino acids 1–140) (SpeI/XhoI) | This study          |
| p423GPD-RTR1-C-V5 | C-terminal V5-tag fusion to C terminus of RTR1 (amino acids 141–226) (SpeI/XhoI) | This study          |
| p416GPD        | Low copy number; strong constitutive promoter | 48                  |
| p426GPD        | High copy number; strong constitutive promoter | 48                  |
| p416GPD-RTR1   | ORF inserted into MCS<sup>a</sup> (SpeI/XhoI) | This study          |
| p426GPD-RTR1   | ORF inserted into MCS (SpeI/XhoI)           | This study          |
| p423GPD-RBP3   | ORF inserted into MCS (SpeI/XhoI)           | This study          |
| p423GPD-RBP4   | ORF inserted into MCS (SpeI/XhoI)           | This study          |
| p423GPD-RBP5   | ORF inserted into MCS (SpeI/XhoI)           | This study          |
| p423GPD-RBP7   | ORF inserted into MCS (SpeI/XhoI)           | This study          |
| p423GPD-RBP8   | ORF inserted into MCS (SpeI/XhoI)           | This study          |
| p423GPD-RBP9   | ORF inserted into MCS (SpeI/XhoI)           | This study          |
| p423GPD-RBP10  | ORF inserted into MCS (SpeI/XhoI)           | This study          |
| p423GPD-RBP11  | ORF inserted into MCS (SpeI/XhoI)           | This study          |
| p423GPD-RBP12  | ORF inserted into MCS (SpeI/XhoI)           | This study          |
| pRPB1         | RPB1 genomic clone                           | N. Woychik          |
| pRPB2         | RPB2 genomic clone                           | N. Woychik          |
| pRS416        | Low copy number; URA<sup>a</sup>            | 59                  |
| p416-RTR1     | Low copy number; native RTR1 promoter       | This study          |
| pRS315 pNOP-GFP-RTR1 | N-terminal GFP fusion (PstI/XhoI)        | This study          |
| pRS315 pNOP-ProA-RTR1 | N-terminal protein A fusion (PstI/XhoI)    | This study          |

<sup>a</sup> MCS, multiple cloning site.

RESULTS

**rtr1Δ** cells are temperature sensitive, whereas overexpression of RTR1 causes a growth defect. In an effort to uncover novel genes required for thermostolerance, we utilized published microarray data to identify unnamed genes induced in response to heat shock (11, 22). In our initial phenotypic characterization of haploid knockouts of these genes, cells carrying a disruption in the YER139C locus were exquisitely sensitive to heat shock-mimetic formamide at 37°C but exhibited no additional stress sensitivities (Fig. 1A). Formamide has been used to exacerbate heat shock phenotypes, because it destabilizes noncovalent bonds in macromolecules (1). Further examination of this temperature sensitivity phenotype showed that rtr1Δ cells have no detectable growth defect at 30°C (doubling time of 1.9 h versus 1.8 h for the wild type), a mild growth defect at 37°C (4.2 h of doubling time), and a severe growth defect at 39°C (6.4 h of doubling time) compared to wild-type cells, which were capable of tolerating growth temperatures up to 41°C (Fig. 1B).

We next examined the consequences of elevated RTR1 expression. Relative growth rates of wild-type cells transformed with an empty vector, a low-copy-number vector expressing RTR1, or a high-copy-number RTR1 expression vector were assessed as shown in Fig. 1C. Increased expression of RTR1 caused a dose-dependent reduction in growth rate: cells carrying an empty vector (low- or high-copy number) grew with a doubling time of approximately 2.1 h, those carrying an extra copy of RTR1 on a CEN plasmid grew with a doubling time of approximately 2.7 h, and those from a 2-μ-based high-copy-number vector grew with a doubling time of approximately 2.7 h.

Rtr1 contains a conserved nonconsensus Zn-finger-like motif essential for function. Analysis of the primary amino acid sequence of Rtr1 revealed a cysteine-rich amino-terminal motif reminiscent of a Zn finger (C-x<sub>4</sub>-C-x<sub>12</sub>-C-x<sub>3</sub>-H; Fig. 2A). However, the spacing and arrangement of the putative Zn-coordinating cysteine and histidine residues are novel, with no precise match to known Zn-finger modules. BLAST analysis revealed that this motif, including the intervening amino acids between the cysteine and histidine residues, is highly conserved in a wide range of eukaryotic species, including fission yeast (Schizosaccharomyces pombe), amoebae (Dictyostelium discoideum), mice, and humans. Homologs are notably absent from both the bacteria and archaea, suggesting that the RTR1 gene family is unique to eukaryotes. The cysteine residues are invariant in these putative homologs, with a high (approximate) degree of sequence identity in the immediately adjacent regions. RTR1 sequence similarity drops to negligible levels among the more distantly related species outside of this con-
proteins. Replacement of either cysteine module completely blocked complementation of the rtr1Δ formamide-temperature sensitivity phenotype (Fig. 2B). To determine whether these rtr1 mutant alleles were stably expressed, cells were grown to logarithmic phase, whole-cell extracts were isolated, and Rtr1 proteins were detected by immunoblot analysis. Both the rtr1(C73S) and the rtr1(C112S, H116S) mutants were produced at wild-type levels (Fig. 2C), demonstrating that lack of complementation was not due to destabilization. Moreover, these results suggest that the putative Zn finger is not required for Rtr1 structural integrity and may instead be involved in protein-protein or protein-nucleic acid interactions.

We next sought to determine whether overexpression toxicity likewise required the cysteine-rich domain. The conserved region comprises approximately half of the 226-amino-acid sequence of the Rtr1 protein. We therefore generated a series of constructs overexpressing the full-length protein or the amino- or carboxy-terminal halves of the protein. For convenience, the small (14-amino-acid) V5 epitope tag was engineered into the amplifying oligonucleotide primers to facilitate evaluation of protein expression. As a control, full-length Rtr1 was synthesized with either an amino- or a carboxy-terminal V5 tag, each of which was inhibitory to growth when overexpressed, as shown in Fig. 2D (see V5-Rtr1 and Rtr1-V5 data; doubling time, 2.7 and 3.1 h, respectively, versus 1.9 h for strains carrying the empty vector). In addition, the overexpressed nonconserved carboxy-terminal half of the protein was equally toxic (Rtr1141-226-V5; 2.8 h doubling time). In contrast, the amino-terminal half of the protein containing the cysteine-rich domain (V5-Rtr11-140) did not result in a growth defect when overexpressed (doubling time, 2.1 h). This mutant protein was stably produced, as detected by immunoblotting (data not shown). Growth inhibition was further exacerbated at 37°C. Together, these data demonstrate that Rtr1 is the yeast homolog of members of a larger eukaryotic gene family that share a highly conserved cysteine-rich motif in the amino terminus that is required for function in vivo. Moreover, the nonconserved carboxy-terminal half of Rtr1 appears to dramatically hinder cell growth when overexpressed in yeast, suggesting the need for a stoichiometric balance of this protein with other cellular components essential for growth under normal and heat shock conditions.

**Rtr1 constitutively shuttles between the cytoplasm and nucleus.** As part of our characterization of the Rtr1 protein, we determined the subcellular localization of Rtr1 by use of a functional GFP-tagged allele expressed from a low-copy-number vector. Under both normal growth conditions (25°C) and elevated temperatures (35°C), GFP-tagged Rtr1 localized within the cytoplasm, in agreement with a previously published proteome-wide localization study (Fig. 3A) (27). We were unable to detect changes in the fluorescence localization pattern upon exposure to other environmental stresses (data not shown). Yeast two-hybrid analysis suggested that Rtr1 may gain access to the nucleocytoplasmic transport system via interaction with Ran (8). Therefore, to test for nuclear localization of Rtr1, GFP-Rtr1 was expressed in cells lacking Xpo1 (Crm1), the major nuclear export factor that shuttles NES-containing proteins out of the nucleus (60). Inactivation of this protein has been shown to lead to accumulation of substrates with rapid transit kinetics (7, 60). Using the temperature-sen-
sensitive allele *xpo1-1*, we observed prominent nuclear localization within 8 min after shifting to the nonpermissive temperature (Fig. 3B). These data demonstrate that Rtr1 constitutively shuttles between cytoplasm and the nucleus and is actively transported out of the nucleus via the Xpo1 system.

Suppression of *rtr1Δ* temperature sensitivity by overexpression of RNAPII core subunits. In an effort to understand the cellular role of Rtr1, we undertook a high-copy-number suppressor screening to identify genes whose overexpression could repair the temperature-sensitive growth defect caused by the loss of *RTR1*. To accomplish this, we transformed a high-copy-number yEP24-based genomic library into the *rtr1*/H9004 background and selected for growth at 37°C in the presence of 2% formamide. Suppressors were then identified and characterized as described in Materials and Methods. From 100,000 independent transformants, we isolated only two independent suppressors of *rtr1*/H9004, RPB5 and RPB9 (Fig. 4). Strikingly, the proteins encoded by these genes are both core subunits of eukaryotic RNAPII. To ask whether this phenomenon was a general property of RNAPII subunit overexpression, we tested 9 of the remaining 10 subunit-encoding genes for high-copy-number suppression of the *rtr1Δ* phenotype. RPB7 was identified as yet another RNAPII subunit suppressor of *rtr1Δ*, while the remaining subunits were unable to confer growth when
overexpressed (Fig. 4). These results suggest that RPB5, RPB7, and RPB9 share a common but unknown functional characteristic that permits overexpression of any one of those genes alone to overcome loss of the Rtr1 protein.

Phenotypic and genetic analyses indicate that Rtr1 has a role in transcription. Because overexpression of RPB9 complements the formamide sensitivity phenotype of rtr1Δ, we considered the possibility that these two proteins are functionally linked. rpb9Δ cells are defective in start-site selection, utilizing secondary transcription start sites for a number of genes. We performed primer extension analysis using the ADH1 gene and observed the reported defects of an rpb9Δ mutant but did not detect obvious transcriptional defects in rtr1/H9004 cells (data not shown). RTR1 is therefore not required for at least this role of RPB9. To further probe the genetic relationship between RTR1 and RNAPII, we tested whether strains lacking RPB9 or the only other nonessential subunit, RPB4, could be sensitized to moderate overexpression of RTR1 by use of the uninduced CUP1 promoter (36). Surprisingly, at a normal growth temperature of 30°C, both rpb9Δ and rpb4Δ cells, but not wild-type cells, were exquisitely sensitive to heightened RTR1 levels (Fig. 5). This effect was completely abrogated when either rtr1(C73S) or rtr1(C112S, H116S), the nonfunctional mutant alleles, was likewise overexpressed. These data, along with the results of the overexpression studies represented in Fig. 2, suggest that Rtr1 exists in an optimal stoichiometric balance with functional RNAPII.

A number of transcription mutants are sensitive to the IMP dehydrogenase inhibitors 6AU and MPA (29, 46, 57). These compounds are thought to decrease the nucleotide pool, thereby inhibiting transcription elongation (54). We tested wild type, rpb9Δ, and rtr1Δ cells for their relative resistances to these inhibitors and, given our previous results, also included formamide. When cells were plated onto media containing no drug, 6AU, MPA, or formamide, we observed differential sensitivity results, as shown in Fig. 6. At drug concentrations that were permissive for the growth of wild-type cells, rtr1Δ cells were sensitive only to MPA at 37°C and were resistant to 6AU. rpb9Δ cells exhibited more severe phenotypes, including sensitivity to 6AU at 37°C and to MPA at both 30°C and 37°C. Interestingly, rpb9Δ and rtr1Δ cells exhibited identical sensitivities to formamide only at 37°C. Loss of RTR1 therefore recapitulates only a subset of the phenotypes exhibited by cells lacking RPB9, specifically in combination with elevated temperature.
To further delineate the transcriptional roles of Rtr1, we tested for conditional synthetic interactions between rtr1Δ and deletion of genes involved in various stages of transcription. We observed a number of synthetic growth defects (SGDs), including inviability in the presence of MPA for rtr1Δ rpb4Δ and rtr1Δ elp2Δ double mutants (Fig. 7A and Table 3). ELP2 encodes a subunit of the Elongator complex, which associates with actively transcribing RNAPII and also possesses histone acetyltransferase activity (21). These interactions were not a general result of transcriptional impairment, as RTR1 exhibited no SGDs with the initiation factors MED1 and GAL11. RTR1 also exhibited substantial interaction with the uncharacterized ORF YDR066C, which, based on the strong level of homology (89% sequence similarity) and the presence of the RTR1 cysteine-rich domain, may be a recently diverged paralog. Two additional initiation factors, SOH1 and SBR5, and the elongation factors CCR4, CDC73, SPT4, and DST1 exhibited modest SGDs. The strongest interactions were observed with the RNAPII core subunits RPB4 and RPB9. In the course of another line of investigation, we also observed a strong temperature-dependent synthetic interaction between rtr1Δ and an allele of Rpb9 that included the TAP tag RPB9-TAP (Fig. 7B). While RPB9-TAP and rtr1Δ were both viable at temperatures of up to 37°C, deletion of RTR1 in the context of RPB9-TAP resulted in an inability to grow at 37°C. These results suggest that the RPB9-TAP allele may be cryptically hypomorphic and further underscore the close relationship between these two proteins.

Rtr1 physically interacts with active Rpb1. Recent large-scale proteomic studies of both yeast and human cells have indicated that Rtr1 (RPAP2 in humans) physically interacts with multiple components of the core RNAPII enzyme (15, 23, 30). Given the extensive genetic interactions we demonstrated between Rtr1 and RNAPII, we sought to validate and extend these findings in more detail. Protein A-tagged Rtr1 was purified as described in Materials and Methods, and copurifying proteins were visualized using Coomassie brilliant blue staining. Rpb1 and Rpb2 were associated with Rtr1, as evaluated by gel migration investigations (Fig. 8A). Immunoblot analysis of the protein A-Rtr1 affinity purification results by use of the specific monoclonal antibody 8WG16 positively identified Rpb1. Additional proteins were coisolated with Rtr1, including a prominent component of about 180 kDa that currently remains unidentified. We noted that Rpb1 migrated as a doublet, as is consistent with previously documented populations of the protein, one unphosphorylated and the other phosphorylated at serines in the second and fifth positions within the highly conserved heptad repeats of the regulatory CTD. Both protein bands were recognized by the 8WG16 antibody that interacts with nonphosphorylated heptad repeats (Fig. 8B) (9, 51, 62). We also tested reactivity of equivalent amounts of the coimmunoprecipitation mixtures with antibodies specific for phosphorylated CTD. The slower-migrating band reacted strongly with the H5 antibody specific for the CTD phosphorylated at serine 5 in the heptad repeat and weakly with the 8WG16 antibody H14 that recognizes phosphorylation at serine 2. These results are consistent with association of Rtr1 and actively transcribing RNAPII. None of the anti-RNAPII antibodies recognized bands from a parallel control TAP purification (Fig. 8B), and Rpb1 and Rpb2 were not detected by Coomassie staining (data not shown), demonstrating the specificity of the Rtr1-TAP · RNAPII interaction.

Rtr1 regulates transcription from the GAL1 promoter. Considering the genetic and biochemical interactions between RTR1 and components of the transcription machinery, we were interested to determine the transcriptional consequences of RTR1 disruption. We examined both inducible gene expression from the GAL regulon and constitutive expression of a standard “housekeeping” gene, ACT1. Because we anticipated that the requirement for RTR1 might be limited to transcription during heat shock, we also examined gene expression after a 1-h shift to 39°C. As shown in Fig. 8C, expression of GAL1 and GAL7 was strongly induced by a shift from sucrose to galactose in wild-type cells but not in rtr1Δ cells at either normal or heat shock temperatures, indicating a significant role for RTR1 in expression from these loci. Expression of ACT1 and the RNAPIII-dependent RNA component of SCR1, the signal recognition particle, was unaffected in rtr1Δ cells. Complementation of rtr1Δ restored GAL1 induction, verifying that loss of transcriptional activity was due to loss of Rtr1 function (data not shown). Interestingly, RNAPII- but not RNAPIII-dependent gene expression appeared to be inhibited at high temperatures, as is consistent with a previous report (67).
This report constitutes the first in vivo characterization of the \textit{RTR1} gene and its protein product. We have elucidated multiple phenotypes associated with loss of \textit{RTR1} and identified a highly conserved amino-terminal motif essential for function. We have further defined multiple genetic and biochemical interactions between \textit{RTR1} and components of the transcription machinery—specifically, subunits of the core RNAPII enzyme. Finally, we observed a defect in transcription from the \textit{GAL1} promoter in \textit{rtr1Δ} cells, demonstrating a functional transcriptional consequence associated with loss of this novel protein. Our analyses indicate that Rtr1 functions in modulating RNAPII-based transcription, specifically via interactions with RNAPII core subunits. These findings are further bolstered by the identification of C1ORF82, the closest human \textit{RTR1} homolog, as an RNAPII-associated factor. This ORF has been renamed RPAP2 (for “RNAPII-associated polypeptide”) and is located within an expansive network of interacting

### DISCUSSION

This report constitutes the first in vivo characterization of the \textit{RTR1} gene and its protein product. We have elucidated multiple phenotypes associated with loss of \textit{RTR1} and identified a highly conserved amino-terminal motif essential for function. We have further defined multiple genetic and biochemical interactions between \textit{RTR1} and components of the transcription machinery—specifically, subunits of the core RNAPII enzyme. Finally, we observed a defect in transcription from the \textit{GAL1} promoter in \textit{rtr1Δ} cells, demonstrating a functional transcriptional consequence associated with loss of this novel protein. Our analyses indicate that Rtr1 functions in modulating RNAPII-based transcription, specifically via interactions with RNAPII core subunits. These findings are further bolstered by the identification of C1ORF82, the closest human \textit{RTR1} homolog, as an RNAPII-associated factor. This ORF has been renamed RPAP2 (for “RNAPII-associated polypeptide”) and is located within an expansive network of interacting

### TABLE 3. Genetic interactions between \textit{rtr1Δ} and other transcription genes

| Gene   | Role (reference) | Result of interaction with \textit{rtr1Δ} under indicated conditions$^a$ |
|--------|------------------|---------------------------------------------------------------------|
|        |                  | YPD 30°C | Form 30°C | MPA 30°C | 6AU 30°C | YPD 37°C | Form 37°C | MPA 37°C | 6AU 37°C | YPD 30°C | Form 37°C | MPA 37°C | 6AU 37°C |
| \textit{GAL11} | Initiation (49) | — | — | — | ND | — | — | — | — |
| \textit{MED1} | Initiation (49) | — | — | — | ND | — | — | — | — |
| \textit{SOH1} | Initiation (41) | — | SGD | SGD | ND | — | SL | — | SGD |
| \textit{SRB5} | Initiation (61) | — | SGD | ND | ND | — | — | — | — |
| \textit{CCR4} | Elongation (18) | — | — | — | ND | — | — | — | — |
| \textit{CDC73} | Elongation (34, 58) | — | — | — | ND | — | SGD | — | — |
| \textit{DST1} | Elongation (50) | — | — | — | ND | — | SGD | — | — |
| \textit{ELP2} | Elongation (21) | SGD | SGD | SGD | ND | SGD | SGD | SGD | SGD |
| \textit{SPT4} | Elongation (42, 55) | — | — | — | ND | — | — | — | — |
| \textit{RPB4} | Core (16) | — | SGD | SGD | ND | SGD | SL | — | SGD |
| \textit{RPB9} | Core (16) | — | SGD | SGD | ND | ND | ND | — | ND |
| \textit{YDR066C} | Unknown | SGD | SGD | SGD | ND | SGD | SGD | SGD | SGD |

$^a$ YPD, rich medium, no stress; Form, 1% formamide; —, no synthetic interaction; ND, not determined (due to single-gene lethality); SL, synthetic lethality.

### FIG. 8. Rtr1 physically interacts with the active form of Rpb1 and regulates transcription from the \textit{GAL1} promoter.

(A) Coomassie brilliant blue staining of a sodium dodecyl sulfate-polyacrylamide electrophoresis gel containing protein A-Rtr1 for affinity purification (described in Materials and Methods). The positions of Rpb1 and Rpb2 are indicated. (B) Immunoblot analysis of Rtr1-TAP and control TAP purifications using anti-Rpb1, anti-Rpb1(Ser5P), and anti-Rpb1(Ser2P) antibodies. (C) Northern blot analysis of wild-type (BY4741) and \textit{rtr1Δ} cells grown to logarithmic phase in sucrose (Suc) medium and either maintained at 30°C or shifted to 39°C for 1 h followed by a shift to galactose (Gal)-containing medium at the indicated temperatures.
protein complexes, as identified by large-scale affinity purification with other transcriptional components (30). However, no functional insights into the roles of RPAP2 have emerged, underscoring the need for further study of the yeast homolog in its in vivo context. For example, comprehensive cataloging of transcriptional defects in rtr1Δ cells would aid the assignment of a specific functional role within the RNAPII multi-protein transcriptional complex.

We believe that the Rtr1/RPAP2 proteins may play a unique role in transcription because of the array of genetic interactions and specific physical interactions with core subunits of RNAPII. While many accessory factors and complexes have been identified (Mediator, Elongator, general and specific transcription factors, etc.), few interact robustly with the core subunits of RNAPII. Eight affinity capture interactions with Rtr1 examined in yeast proteome-wide analyses have been described previously—among them, interactions with the nucleolar protein Rpb2, with the microtubule-associated protein Bik1, and with the enzyme Ura2, an apparently promiscuous binding protein with 43 unconfirmed affinity interactions (23, 24, 33). The remaining four Rtr1 interactors are RNAPII subunits: Rpb1, Rpb2, Rpb3, and Rpb8 (15, 23). Strikingly, RPAP2 likewise interacts with at least eight core subunits (30).

We confirmed the interaction of Rtr1 with Rpb1 in yeast cells and showed that Rtr1-associated Rpb1 is transcriptionally active. Further, RTR1 genetically interacts with transcriptional components acting at multiple stages in transcription, including initiation and elongation. Core RNAPII subunits RPB4 and RPB9 exhibited some of the strongest genetic interactions with rtr1Δ. In addition, the RPB9-TAP allele also rendered cells temperature sensitive in the context of rtr1Δ. The heat shock/ formamide sensitivity phenotype is shared by a number of other transcriptional mutants, including those defective in the Pafl complex (Ctr9, Cdc73, Ccr4, Hpr1, Rtf1, Leol) (12). RPAP2 also reappears to associate with at least eight additional polypeptides in one or more complexes, many of which have yeast counterparts (30). When taken together, these numerous genetic and biochemical interactions place Rtr1/RPAP2 as part of the DNA clamping mechanism of transcriptional defects in rtr1Δ and ydr066cΔ, indicating possible functional redundancy between the two proteins (Table 3). We therefore propose renaming the uncharacterized ORF YDR066c RTR2, although at this time we do not have evidence for direct interaction with RNAPII.

Zn-finger motifs are predominantly involved in nucleic acid binding; as a result, the spacing between cysteine and histidine residues contributes to binding specificity (37). However, unlike many Zn fingers, the residues between the putative metal chelating cysteines and histidine in Rtr1 are also highly conserved. It may also be noteworthy that RPB5 and RPB9, two of the three high-copy-number suppressors, are themselves Zn-containing proteins (19, 63). In fact, RPB9 is a small 122-amino-acid subunit that harbors two distinct Zn-binding domains organized into what has been termed a “zinc ribbon.” Because RPB5 and RPB9 are both located at the “jaws” of RNAPII and function as part of the DNA clamping mechanism, it is tempting to speculate that Rtr1 may function as an accessory DNA binding factor for RNAPII in a mechanism requiring its cysteine-rich motif. Further analysis of the precise protein-protein interactions of Rtr1/RPAP2 with RNAPII, of its presence or absence on the actively transcribing enzyme, and of gene-specific transcriptional requirements are needed to provide a full understanding of this protein family.

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