Rct1, a Nuclear RNA Recognition Motif-Containing Cyclophilin, Regulates Phosphorylation of the RNA Polymerase II C-Terminal Domain^{∇†}

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Phosphorylation of the C-terminal domain (CTD) of RNA polymerase II (RNAP II) is a dynamic process that regulates transcription and coordinates it with pre-mRNA processing. We show here that Rct1, a nuclear multidomain cyclophilin from *Schizosaccharomyces pombe*, is encoded by an essential gene that interacts with the CTD and regulates its phosphorylation in vivo. Downregulation of Rct1 levels results in increased phosphorylation of the CTD at both Ser2 and Ser5 and in a commensurate decrease in RNAP II transcription. In contrast, overexpression of Rct1 decreases phosphorylation on both sites. The close association of Rct1 with transcriptionally active chromatin suggests a role in regulation of RNAP II transcriptional activity. These data, together with the pleiotropic phenotype upon Rct1 deregulation, suggest that this multidomain cyclophilin is an important player in maintaining the correct phosphorylation code of the CTD and thereby regulating CTD function.

Eukaryotic RNA polymerase II (RNAP II), the enzyme responsible for transcription of protein-coding genes, is a multisubunit complex. For accurate transcription, RNAP II is controlled by many factors through protein-protein interactions. RNAP II transcripts undergo extensive processing, including capping, splicing, and polyadenylation, which are tightly coordinated with transcription. There is now strong evidence that RNAP II coordinates these processing reactions and couples them to transcription. Specifically, the C-terminal domain (CTD) of the largest RNAP II subunit has a critical role in targeting pre-mRNA processing factors to emerging pre-mRNAs. Thus, the CTD coordinates transcription with pre-mRNA processing and is therefore subject to tight regulation during the transcription cycle (1, 17, 35, 37, 39, 41, 46, 53).

In all eukaryotes, the CTD consists of variable numbers of YSPTSPS heptapeptide repeats. During the transcription cycle, the CTD undergoes dynamic phosphorylation and dephosphorylation on Ser2 and Ser5. Transcription initiation requires an unphosphorylated CTD. Following initiation, the CTD becomes phosphorylated at Ser5, a modification that is necessary to initiate transcript elongation. Finally, extensive phosphorylation at Ser2 is accompanied by transcript elongation and termination. In addition to regulating RNAP II transcription, the CTD phosphorylation status is also important for coordination of transcription with pre-mRNA processing, in particular for recruitment of RNA-processing factors to nascent transcripts. The CTD

associates in a phosphorylation-dependent manner directly or indirectly with a variety of pre-mRNA-processing factors, including 5' capping enzymes, splicing factors, and some components of the 3' end-processing machinery cycle (1, 17, 35, 37–39, 41, 46, 53).

Recently, *cis-trans* isomerization of prolyl peptide bonds in the heptapeptide repeats has been revealed as an additional level of regulation of CTD structure and function, as it has been shown that three CTD-interacting proteins bind to the CTD only in an all-*trans* proline conformation (10, 31, 32, 34, 48). One of these proteins is the parvulin-type peptidyl-prolyl *cis-trans* isomerase (PPIase) Pin1, which has previously been shown to be important for keeping the correct CTD phosphorylation status in mouse and human cells (51, 52). In addition, mutation of ESS1, a *Saccharomyces cerevisiae* homolog of Pin1, resulted in defects in pre-mRNA 3' end formation (14). These data suggest that enzymes catalyzing *cis-trans* isomerization of proline peptide bonds might be important for correct CTD conformation and phosphorylation and consequently for its recognition by diverse pre-mRNA-processing factors. Thus, generation of the CTD code requires not only phosphorylation but also *cis-trans* isomerization of the two prolines that follow Ser2 and Ser5 in the heptapeptide repeats (3, 24, 32, 34).

The majority of cyclophilins (PPIases) are small proteins containing only a PPIase domain of about 120 amino acids. However, several multidomain cyclophilins from different organisms have been described as well (36, 42). The most complex multidomain cyclophilins characterized thus far are *Arabidopsis* AtCyp59 (12) and its orthologue from *Paramecium tetraurelia*, Kin241p (22). They are characterized by a unique domain organization, consisting of a PPIase domain at the N terminus, followed by an RNA recognition motif (RRM) and a C-terminal domain enriched in charged amino acids and serines or RS/RD dipeptide repeats. Homologous proteins

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have been found in the majority of eukaryotes, although *Saccharomyces cerevisiae* and some other *Saccharomycotina* species do not encode this kind of protein (12, 22). Kin241p was identified in *Paramecium tetraurelia* as a protein involved in cell morphogenesis (reference 22 and references therein). AtCyp59 is a nuclear protein and was identified in a yeast two-hybrid screen as an interacting partner of SR proteins, an important family of splicing regulators (43). As it also interacted with the CTD of RNAP II, a function for this protein at the interface between transcription and pre-mRNA splicing was proposed (12). Interestingly, *Arabidopsis* cells ectopically expressing AtCyp59 are characterized by very slow growth, presumably due to a decreased phosphorylation of the RNAP II CTD (12). As no *Arabidopsis* mutant in the gene encoding AtCyp59 exists, and as no homologous protein is present in *S. cerevisiae*, we switched to an alternative genetically treatable organism, *Schizosaccharomyces pombe*. The *S. pombe* homolog is 49% identical and 66% similar to AtCyp59, with the same domain organization as AtCyp59 (see Fig. 1A) except for a Zn knuckle which is located downstream of the RRM in all plant homologs (12). This sequence conservation also suggests a highly conserved function.

Here, we show that Rct1 (for “RRM-containing cyclophilin regulating transcription”), the *S. pombe* ortholog of *Arabidopsis* AtCyp59, is encoded by an essential gene. Reduced levels of Rct1 in *rct1* heterozygous cells result in strong growth and morphological defects and have an impact on meiotic differentiation. Furthermore, phosphorylation of the CTD is increased and ongoing transcription of RNAP II is reduced. Most of the observed phenotypes are rescued by complementation assays. In addition, higher levels of Rct1 resulted in decreased phosphorylation of the CTD and again in reduced growth. Its close association with the active transcription complex places this essential protein at a central position in regulating CTD phosphorylation throughout the transcription cycle. Together with recent structural data on CTD-interacting proteins, our data emphasize the importance of PPIases in the formation of the CTD code.

MATERIALS AND METHODS

***S. pombe* strains and handling of cells.** The diploid *S. pombe* strain P136 (*h+*/*h*– *leu1-32/leu+* *ura4-27/ura+* *ade6-704/ade+*) was used as the wild-type (WT) strain for all experiments. If not stated otherwise, all WT strains (WT, WT^{pMG} and WT^{pMG1}) used are diploid. Media, growth conditions, and standard genetic methods used throughout were as described previously (33; <http://www-rcf.usc.edu/~forsburg>). Geneticin (G418; Sigma) and ClonNat (BioAgents) were used at final concentrations of 155 and 100 µg/ml, respectively. The *mtl1* promoter was repressed by the addition of thiamine (final concentration, 100 µg/ml).

Construction of *rct1* knockout allele and plasmids and generation of *rct1*^{+/+}pMG1, *rct1*^{+/+}pMG1^Δ, and *rct1Δ*^{pMG1} strains. Fragments corresponding to the 5' and 3' untranslated regions (UTR) of the *rct1* gene were amplified by PCR. For the 5' UTR, oligonucleotides 5'-GACTAGGGATCCTCTAGAAACGAATGTATCA CATTGC-3' (forward) and 5'-GACTAGAAGCTTTTCAATTAGTACAGAC ATG-3' (reverse), which introduce a BamHI and a HindIII site, respectively, were used. For the 3' UTR, oligonucleotides 5'-GACTAGCTGCAGCATCGT GACGATCAAGTTCA-3' (forward) and 5'-GACTAGTCTAGACAAGTAT AAGCAATGTAGATTTC-3' (reverse), which introduce a PstI and an XbaI site, respectively, were used. PCR fragments were cloned into pClonNat1. The pClonNat15'UTR3'UTR construct was then linearized with XbaI and transformed into WT cells by the lithium acetate method. Correct integration of pClonNat15'UTR3'UTR into the *rct1* gene was analyzed by Southern blotting. Heterozygous *rct1*^{+/+} cells were grown on malt extract sporulation medium plates for 3 days at 26°C, and tetrads were dissected with a Singer micro-

manipulator. Spores were grown on yeast extract-supplement (YES) medium for 4 days at 32°C.

pMG was derived from pREP3X (11) by inserting a kanamycin resistance cassette, cut out from pSMRG2+ (25), into the SacI site. The plasmid expressing Rct1 fused to a hemagglutinin (HA) tag was constructed by cloning the *rct1* gene, amplified by PCR with the oligonucleotides 5'-GTCAGTCTCGAGATGTCTG TACTAATTGAA-3' (forward) and 5'-GTCAGTGTGCACTCATGCGTAGTC AGGCACATCATACGGATATCGATATCTATCATCTCTATAACG-3' (reverse). The PCR fragment was ligated into pMG opened with XhoI/Sall, resulting in pMG1.

To generate different cell strains with the *rct1*-HA gene expressed from the plasmid, WT and *rct1*^{+/+} cells were transformed with pMG1. The haploid *rct1Δ*^{pMG1} strain (*h*– *leu+* *ura+* *ade6-704*) was obtained from *rct1*^{+/+}pMG1 cells after tetrad dissection.

Conditions for PCR genotyping. Loopfuls of freshly growing cells were transferred into Eppendorf tubes and boiled in a microwave for 3 min. Cells were then resuspended in 50 µl of sterile water and used as a template for PCR under the following conditions: 95°C for 3 min; 30 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 1 min; and once at 72°C for 5 min. The presence of WT *rct1* and disrupted *rct1Δ*:ClonNat alleles was checked by PCR by using the oligonucleotide pairs 5'-GAGGCAGAAGCAGAGGCTGTACA-3' (forward)–5'-AACGTG CCGCATTTATGGAG-3' (reverse) and 5'-GCGTGGGGACAATTCAACGC-3' (forward)–5'-AACGTGCCGCAATTATGGAG-3' (reverse), respectively. The presence of the pMG1*Rct1*-HA construct in cells was analyzed by PCR by using the oligonucleotides 5'-GGAATCCGATTGTCATTCCGC-3' (forward) and 5'-CCCCGGGATCCTCTAGAGTC-3' (reverse).

Nuclear transcription run-on analysis. Gene Screen Plus membrane (Perkin-Elmer) was soaked in 1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and fixed in a MINifold slot blot apparatus (Schleicher and Schuell) to apply the DNA. Ten micrograms of double-stranded DNA prepared by PCR was denatured and applied per slot. DNA was fixed to membranes by UV cross-linking in a Stratalinker 2400 (Stratagene). Membranes were prehybridized in hybridization buffer (10 mM Tris-HCl [pH 7.5], 250 µg tRNA, 10× Denhardt's solution [0.2% bovine serum albumin, 0.2% polyvinylpyrrolidone {molecular weight, 40,000}, 0.2% Ficoll 400], 0.5% nonfat dry milk, 0.3 M NaCl, 1% sodium dodecyl sulfate [SDS], 10 mM EDTA) for 2 h at 60°C. *S. pombe* WT and *Rct1*^{+/+} cells were grown in YES medium until reaching exponential phase. The optical density at 600 nm (OD₆₀₀) was measured, and the same amount of cells was harvested by centrifugation at 4,000 rpm for 5 min at room temperature. Pellets were washed with 1 ml of ice-cold TMN buffer (10 mM Tris-acetate [pH 7.0], 12 mM MgCl₂, 50 mM NH₄Cl) and resuspended in 0.5 ml of TMN buffer and equilibrated for 10 min on ice. TMN buffer was then removed, and pellets were resuspended in 950 µl of cold water and 50 µl of 10% sodium sarcosyl and incubated on ice for an additional 20 min. Detergent was removed, and permeabilized cells were resuspended in 0.5 ml of run-on buffer (50 mM Tris-HCl [pH 7.9], 80 mM MgCl₂, 500 mM KCl, 1 mM dithiothreitol [DTT], 1 mM ATP, 0.5 mM GTP, 0.5 mM CTP, 100 units of RNasin, and 100 µCi of [³²P]UTP). The protocol used can be found at <http://www.hsph.harvard.edu/wolflab/Protocols/Protocols/Fission%20Yeast/Nurse%20Lab%20Manual.htm>. Cells were incubated at 30°C for 10 min and washed with TMN buffer, and total RNA was isolated by using TRIzol reagent (Invitrogen). Isolated RNA was dissolved in 100 µl of Tris-EDTA (TE), denatured for 3 min at 95°C, and added to the prehybridized membrane in 3 ml of hybridization buffer. Hybridization was performed at 60°C for 2 days. Membranes were then washed twice with 2× SSC–1% SDS for 10 min at 60°C and exposed to MR Kodak films. In addition, blots were analyzed by PhosphorImaging and quantified by ImageQuant, version 5.1. A list of genes analyzed by nuclear transcription run-on and oligonucleotides used for amplification of corresponding DNA fragments, as well as for reverse transcriptase PCR (RT-PCR) (see below), is available in Table S1 in the supplemental material. Fragment sizes were 400 to 500 nucleotides (nt) and originated from the middle of the coding region. In case of U1 snRNA, U6 snRNA, and tRNA^{Ser}, amplified DNA fragments cover the entire sequences of the mature RNAs.

RNA isolation, RT-PCR, and real-time PCR. Total cellular RNA from *S. pombe* cells in exponential phase was isolated with TRIzol (Gibco). RNA was dissolved in the proper amount of sterile water and treated with RNase-free DNase (Promega) for 30 min at 37°C. One microgram of total RNA was used for reverse transcription, which was performed according to the manufacturer's instructions (Reverse Transcription System; Promega) by using oligo(dT)₁₅ primer. cDNA was diluted to 100 µl with TE buffer, and 10 µl was used for PCR. Conditions for PCRs were 1 cycle at 95°C for 3 min; 30 cycles at 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min; and 1 cycle at 72°C for 5 min. Oligonucleotides used for amplification of corresponding DNA fragments (genes 1 to 10 in Fig. 5B) are available in Table S1 in the supplemental material. Conditions for

RT-PCR amplification of meiotically induced genes were exactly as described previously (2). Oligonucleotides used for amplification of meiosis-unrelated genes *nak1*, (AF091345), *hob1* (AF275637), and *wsp1* (AF038575) were as follows: *nak1*for, 5'-CTGGAATGTCAAGCTATGTG-3'; *nak1*rev, 5'-CCTGCA AACAAGAGGCAATG-3'; *hob1*for, 5'-CAAAGCGTGAAGAAGCAGCA-3'; *hob1*rev, 5'-ACCTTGTGCACCGTTAAGTC-3'; *wsp1*for, 5'-CGATAGCTGA ATTGCTCAAC-3'; *wsp1*rev, 5'-ATCGTCTGCTTCATCCTCTTC-3'. Conditions for PCRs were 1 cycle at 95°C for 3 min; 30 cycles at 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min; and 1 cycle at 72°C for 5 min. PCR products were analyzed on 1.5 to 2% agarose gels.

Real-time PCRs were done with the Corbett Research Rotor-Gene GG-3000 machine. All samples were run in triplicate to ensure accuracy of the data. PCR of 45 cycles was done with QuantiTect SYBR green PCR Master Mix (QIAGEN), 2 µl of precipitated DNA, and 1 µM oligonucleotides. Cycling parameters were 95°C for 15 min followed by 45 cycles of 94°C for 2 s, 55°C for 11 s, and 68°C for 20 s. Oligonucleotides used were as follows: *spo4*PROfw, 5'-TGGTTATGACAGGTGT GCTG-3'; *spo4*PROrev, 5'-CCATGATCATCATTAACCG-3'; *spo4*ORFfw, 5'-ATGCTACATGAACACGAGG-3'; *spo4*ORFrev, 5'-GGGTGTAAGGATTC AAGCA-3'; *spo4*TERfw, 5'-TTGTATCACTTAACCTCTAA-3'; *spo4*TERrev, 5'-ATGACTTGACGCTACAGGCAT-3'; *meu13*PROfw, 5'-CCCTTCGCAAGGTTG GAAA-3'; *meu13*PROrev, 5'-ACTAAGGAAGAAAATCCATG-3'; *meu13*ORFfw, 5'-GCTTTAAATAACTCACTCAG-3'; *meu13*ORFrev, 5'-TTCCAAGGAATCC GTAA-3'; *meu13*TERfw, 5'-GCAGACAAGTCTCTAATAT-3'; *meu13*TERrev, 5'-TCATATCCGTTCCGAAATT-3'; *mfr1*PROfw, 5'-GTGGTCAACTGATCAT TGTT-3'; *mfr1*PROrev, 5'-TGAAACACAGGTGGCTTGA-3'; *mfr1*ORFfw, 5'-TAATGTCTTGGCAGTCGGAC-3'; *mfr1*ORFrev, 5'-TTTCGTAGACTCGATA TCC-3'; *mfr1*TERfw, 5'-CAATCATCTCAACCCCATCA-3'; *mfr1*TERrev, 5'-A ATACAGCCTCAAGCCTGAA-3'; *mei2*PROfw, 5'-CTCCTCTCTAAGCTTTG TT-3'; *mei2*PROrev, 5'-ACAATAAAGCTGGCCGATAA-3'; *mei2*ORFfw, 5'-CC GCTAAATCACTGCGATCT-3'; *mei2*ORFrev, 5'-GTGGACCGAAGCTGCTGA AGT-3'; *mei2*TERfw, 5'-CGAAGAGATCCTGTATTG-3'; *mei2*TERrev, 5'-GA TACTCAGATGAAGTTGA-3'; *act1*PROfw, 5'-GGTGGTATGAAGCCGTTG ATTAC-3'; *act1*PROrev, 5'-TTCTGCCGTGAAGTGCTA-3'; *act1*ORFfw, 5'-TG GGAACAGTGTGGGTAAACA-3'; *act1*ORFrev, 5'-AGCACCTTGCTTGTGTA CT-3'; *act1*TERfw, 5'-CCGGACTCGAGAAGAAACAT-3'; *act1*TERrev, 5'-AAC CACCTTTTCCGCTCTT-3'. Fluorescence intensities were plotted against the number of cycles by using an algorithm provided by the manufacturer.

Cell wall and actin staining, immunofluorescence, and microscopy. Cell wall growth was analyzed by staining living cells with aniline blue. Actin staining was performed as described (5, 6) by using Alexa Fluor 488-phalloidin (Molecular Probes). Immunofluorescence was performed exactly as described previously (40). Rat anti-HA monoclonal antibody (3F10; Roche) was used at a 1:200 dilution. Secondary antibody was goat anti-rat antibody–Alexa Fluor 563 (Molecular Probes) at a 1:200 dilution. DNA was visualized by Hoechst 33342 (Molecular Probes) staining. If not stated otherwise, cells were grown until mid-exponential phase in YES and EMM media and analyzed by microscopy (Axioplan epifluorescence microscope; Zeiss) with differential interference contrast (DIC) optics and 63× oil objective. Images were acquired by a charge-coupled-device (CCD) camera and further processed with Adobe Photoshop.

Immunoprecipitation. *rct1*^{+/−}PMG1 cells were grown in 25 ml of EMM for 15 h, collected by centrifugation, and washed once with ice-cold water. Cells were resuspended in 500 µl of PEB400 buffer (50 mM HEPES-KOH [pH 7.9], 400 mM KCl, 2.5 mM MgCl₂, 1 mM EDTA, 1 mM DTT, 0.1% Triton X-100) (26), supplemented with EDTA-free protease inhibitor cocktail (Roche). Two cell volumes of glass beads were added, and cells were vortexed three times for 15 s. Finally, cells were sonicated once and subsequently centrifuged at 4°C for 15 min at 13,000 rpm in an Eppendorf centrifuge. Supernatant was mixed with 500 µl of PEB without KCl, and immunoprecipitation was done with anti-HA antibody, as described previously (26).

Chromatin immunoprecipitation. Chromatin immunoprecipitation experiments were done as described previously (47). Precipitated DNA was resuspended in 100 µl of TE buffer, and 10 µl was used for PCR. Conditions for PCRs were 1 cycle at 95°C for 3 min; 31 cycles at 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min; and 1 cycle at 72°C for 5 min. PCR products were analyzed on 1.5 to 2% agarose gels. Oligonucleotide sequences used for the PCRs shown in Fig. 4A are available upon request.

SDS-PAGE and Western blotting. Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (10% gels) and transferred to polyvinylidene difluoride membranes (Millipore), followed by Western blotting according to standard procedures. Rat anti-HA (3F10) (Roche), mouse antitubulin (Sigma-Aldrich), and mouse anti-CTD monoclonal antibodies (H14, H5, 8WG16; Covance) were used at 1:1,000 dilutions. Anti-Rct1 polyclonal antibodies were raised in rabbits immunized simultaneously with two peptides, P1 (H₂N-DLVE

PLRSPSTPEQC-COOH) and P2 (H₂N-CERRYRYDRYRDDRYR-COOH) (Eurogentec). Antibodies were affinity purified on a SulfoLink (Pierce) column coupled with both peptides. After purification, antibodies were concentrated to 1 µg/µl and were used at 1:1,000 dilutions. Secondary antibodies, goat anti-rat (Sigma-Aldrich) and goat anti-mouse (Bio-Rad) immunoglobulin G (IgG) and goat anti-mouse (Sigma-Aldrich) IgM, all conjugated with horseradish peroxidase, were used at 1:10,000 dilutions. A chemiluminescence kit (Amersham Pharmacia Biotech) was used for developing the blots.

RESULTS

Rct1, encoding the *Schizosaccharomyces pombe* ortholog of AtCyp59, is an essential gene. To analyze knockout mutants of *rct1*, we performed *rct1* gene disruption in a diploid *S. pombe* strain, as confirmed by Southern blotting and by PCR genotyping (data not shown). Integration of pClonNat, containing a selectable antibiotic gene, resulted in heterozygous cells containing one *rct1*Δ:ClonNat and one *rct1* allele. These cells are hereafter referred to as heterozygous *rct1*^{+/−} or short *rct1*^{+/−} cells. Tetrad dissection from *rct1*^{+/−} heterozygous diploid cells resulted in two growing spores (Fig. 1B). PCR genotyping revealed the presence of only a WT allele in all growing spores. In addition, none of the viable spores was resistant to ClonNat, further indicating that they were WT and that *rct1* is an essential gene. Microscopic analysis of nongrowing spores showed that spores deleted for *rct1* germinate but cell division stops after few generations. We failed to grow these cells even at different temperatures or in liquid medium (data not shown). Together, these results indicate that *rct1* disruption is lethal and not temperature sensitive.

The cellular localization of Rct1 was analyzed by indirect immunofluorescence in *rct1*^{+/−} or *rct1*Δ strains harboring a plasmid expressing Rct1 fused to a HA tag (pMG1; see below). Cells were grown for 18 h in minimal medium (EMM) to derepress the *nmt1* promoter driving transcription of Rct1-HA from the plasmid, followed by fixation and indirect immunofluorescence with anti-HA antibody. As shown in Fig. 1C (shown are only *rct1*Δ^{pMG1} cells), anti-HA antibody detected Rct1-HA only in the nucleus. Staining of fixed cells with Hoechst showed that the Hoechst and Alexa 563 signals are overlapping (Fig. 1C). Analysis of mitotic cells revealed that Rct1 enters new nuclei very rapidly after division, prior to septum formation (Fig. 1C, two lower panels).

Disruption of one *rct1* allele results in growth, morphological, and meiotic defects that are rescued by episomal expression of Rct1. Because we observed that *rct1*^{+/−} cells grow more slowly than do WT cells, we decided to analyze *rct1*^{+/−} cells more thoroughly. Microscopic analyses revealed that *rct1*^{+/−} cells are much shorter and slightly thicker than WT cells. The same phenotype was observed with cells grown on rich medium (YES) plates or in liquid YES medium (Fig. 1D). Growth analysis in YES medium revealed that *rct1*^{+/−} cells grow considerably more slowly than do WT cells (Fig. 1E). Growth rates in EMM were also affected, though not to the same extent as in YES (data not shown). The growth rate of an unrelated knockout strain (termed 93) was comparable to that of WT cells, indicating that the slower growth of *rct1*^{+/−} cells is not due to the presence of ClonNat in the medium. Surprisingly, cells grown overnight in minimal medium without a nitrogen source (EMM-N) strongly sporulated even at 32°C (Fig. 1D). Sporulation was highly enhanced compared to WT cells, and

FIG. 1. Analysis of *rct1*^{+/-} cells. (A) Schematic representation of the domain structure of Rct1. (B) Tetrad analysis of *rct1*^{+/-} diploid *S. pombe* cells. Six asci were dissected, and spores were grown on YES plates at 32°C for 5 days. (C) Rct1 is a nuclear protein. *rct1*Δ^{PMG1} cells were grown overnight in EMM and processed for immunostaining with anti-HA antibody. Rct1 localizes into the nuclei of interphase cells (upper panels) as well as of dividing cells (two lower panels). Hoechst staining was performed to determine the position of nuclei, and DIC images were acquired to determine cell shapes. (D) Microscopic analysis of WT and *rct1*^{+/-} cells grown overnight in liquid YES and EMM media as well as of cells grown for 2 days on YES plates. Images were acquired with a CCD camera by using DIC optics and a 63× oil objective. (E) Growth analysis of WT, *rct1*^{+/-}, and the unrelated *S. pombe* 93 strains. Cells were grown overnight in YES medium and diluted to an OD₆₀₀ of 0.1 in YES medium, and the OD₆₀₀ was measured every 2.5 h. (F) Analysis of Rct1 levels in WT and *rct1*^{+/-} cells. Analysis of the tubulin level (right) was used as a loading control.

virtually all cells were sporulated after 34 h, whereas sporulation of WT cells was around 50% (Fig. 2D and data not shown).

WT *S. pombe* cells are rod-shaped (cylindrical), and their polarity is expressed in the pattern of cell growth, which is restricted to the ends of the long axis of the cell (16, 29). In WT cells grown in YES and EMM, both aniline blue staining and actin localization revealed patterns characteristic of dividing asynchronous cells; the strongest staining was at the cell tips and at sites of septum synthesis. In contrast, many *rct1*^{+/-} cells were stained asymmetrically either on one cell end or between cell tips (aniline blue). In addition, in *rct1*^{+/-} cells, actin was found throughout the cell or in curved cables running either along the long axis of the cell or curling around the nucleus or the cell surface (see Fig. S1 in the supplemental material). All growth and morphological changes with *rct1*^{+/-} cells were observed either in the presence or absence of ClonNat, indicating that they are not due to the presence of the antibiotic. Therefore, we compared Rct1 protein levels in WT and *rct1*^{+/-} cells and found that Rct1 levels are considerably lower in *rct1*^{+/-} cells (Fig. 1F, left), obviously leading to haploinsufficiency.

To confirm that the observed phenotypes in *rct1*^{+/-} cells are indeed a consequence of the reduced levels of Rct1, in vivo complementation was performed. A plasmid expressing HA-tagged Rct1 (pMG1) under control of the thiamine-repressible *mnt1* promoter (Fig. 2A, top) was transformed into *rct1*^{+/-} diploid cells, forming the *rct1*^{+/-}-pMG1 strain. We also gener-

ated a potential overexpressing strain, WT^{pMG1}, and, as a control, a WT^{pMG} strain by transforming WT diploid cells with plasmids pMG1 (tagged Rct1) and pMG (empty vector), respectively. Tetrad dissection of *rct1*^{+/-}-pMG1 cells resulted in only two growing spores. PCR genotyping, as well as growth in the presence of ClonNat and G418, revealed that they are *rct1*Δ haploid cells harboring pMG1 (hereafter *rct1*Δ^{pMG1}). Although the *mnt1* promoter is repressed by thiamine present in YES medium and should be off, it is still active to some extent, and detectable amounts of protein are produced (Fig. 2A). The *rct1*Δ^{pMG1} cells grown overnight in YES or EMM were of the size and shape expected for haploid WT cells (Fig. 2C, top, and Fig. S1 in the supplemental material), although some irregularly shaped cells were observed as well (data not shown; also see below). This indicates that low expression of Rct1 from the plasmid in YES medium (Fig. 2A) is sufficient to complement *rct1*Δ cells. Why we never recovered WT spores expressing additional Rct1 from the plasmid is unclear.

Growth analysis showed that *rct1*^{+/-}-pMG1 and *rct1*Δ^{pMG1} grow at approximately the same rate, somewhat slower than WT but faster than *rct1*^{+/-} (Fig. 2B). The addition of thiamine (100 μg/ml) significantly reduced the growth of *rct1*Δ^{pMG1} cells, but, unexpectedly, it did not stop it completely (Fig. 2B). Analysis of the Rct1-HA protein in these cells revealed the presence of low levels of HA-tagged Rct1 even 24 h after the addition of thiamine (Fig. 2C, insert in the left bottom panel). This explains why haploid *rct1*Δ^{pMG1} cells were still growing

FIG. 2. Episomal expression of Rct1 complements *rct1*^{+/-} cells. (A) (Top) Schematic presentation of plasmid pMG1, expressing HA-tagged Rct1. The *nmt1* promoter is repressed by thiamine and is therefore active only in minimal medium (EMM), which does not contain thiamine. (Bottom) Expression analysis of Rct1-HA in *rct1*^{+/-}pMG1, *rct1*ΔpMG1, and WTpMG1 cells grown overnight in EMM (left) or YES (right) medium. Western blotting was performed with anti-HA antibody. The lower right panel represents an analysis of Rct1 levels; the band marked with an asterisk represents an antibody cross-reacting peptide. (B) Growth analysis of WT, WTpMG1, WTpMG1, *rct1*^{+/-}, *rct1*^{+/-}pMG1, and *rct1*ΔpMG1 cells in liquid YES medium. Cells were grown overnight in YES medium, diluted to an OD₆₀₀ of 0.1, and further grown for 25 h in the presence of ClonNat and G418. *Rct1*ΔpMG1 cells were also grown in the presence of thiamine (*rct1*ΔpMG1 + T) to inhibit expression of Rct1-HA from plasmid. (C) Actin, DNA, and cell wall (Hoechst and aniline blue [H+AB]) staining of *rct1*ΔpMG1 cells grown overnight in YES (top) or for 24 h in the presence of thiamine (bottom). Note mycelial, branched, and otherwise unseparated *rct1*ΔpMG1 cells and asymmetric aniline blue staining (AB). Cells in the top and bottom panels (on the left) are at the same scale. All cells in the right panel are at the same scale. Images were acquired with a CCD camera by using DIC optics and a 63× oil objective. The insert in the bottom-left panel represents an analysis of the Rct1-HA level in *rct1*ΔpMG1 cells grown in the presence of thiamine. (D) Analysis of sporulation defects of *rct1*^{+/-} cells. WT, *rct1*^{+/-}, *rct1*^{+/-}pMG1, *rct1*^{+/-}pMG, and WTpMG1 cells were inoculated in EMM-N medium at an OD₆₀₀ of 0.1 and grown at 32°C for 24 h. Sporulation was scored after 24 h by counting 100 cells for each strain. (E) RT-PCR analysis of meiotically induced genes from cells grown in EMM-N. Samples were taken at the indicated time points, and RT-PCR was performed as described previously (2). Positions of unspliced and spliced transcripts are indicated on the right.

and also shows that very low levels of Rct1 are able to support some cell growth. However, the *rct1* Δ^{pMG1} cells grown for 24 h or longer in the presence of thiamine in both media became wider (swollen) and much shorter than WT cells, with a variety of growth shapes ranging from normal and rod-like to rounded, pear-like, irregularly branched, or highly elongated (Fig. 2C and data not shown). Also, many cells grow in short chains, indicating defects in cell separation. It is also worth noting that WT^{pMG1} cells that overexpress Rct1 grow very slowly, in accordance with our previous data on AtCyp59 (12), although no morphological defects were observed. Finally, the results shown in Fig. S1 in the supplemental material clearly indicate that all other phenotypes observed in *rct1*^{+/−} cells are rescued by episomal expression of Rct1-HA.

During their life cycle, diploid *S. pombe* cells heterozygous for the mating-type genes can either undergo mitotic proliferation or enter meiosis. The decision between these two developmental states depends on nutrient availability, particularly nitrogen, and on growth temperature. We show that the regulation of meiotic differentiation is perturbed in *rct1*^{+/−} cells grown at 32°C in EMM-N (EMM without a nitrogen source) (Fig. 1D). In addition, we show that genes important for the entrance into meiosis were up-regulated in *rct1*^{+/−} cells and demonstrated activated splicing (Fig. 2E). Ectopic expression of Rct1-HA in these cells (*rct1*^{+/−}pMG1 strain) resulted in complementation of this defect. Interestingly, however, after 24 h in EMM-N, only ~15% of *rct1*^{+/−}pMG1 cells sporulated, compared to ~35% of WT cells. Similarly, overexpression of Rct1 in the WT background (WT^{pMG1}) also reduced sporulation efficiency (Fig. 2D). Together, these data indicate that precise regulation of Rct1 levels is necessary for correct meiotic differentiation. We also analyzed three spliced transcripts that are not induced by meiosis (*nak1*, *hob1*, and *wsp1*). As shown in Fig. 2D, their splicing was not affected by underexpression of Rct1, indicating that Rct1 is most probably not a general splicing regulator.

CTD phosphorylation status is affected in *rct1*^{+/−} cells. Our previous results obtained with the *Arabidopsis* Rct1 homolog, AtCyp59, showed that it interacted with the CTD of RNAP II and that ectopic expression of AtCyp59 in cell suspension culture resulted in a highly reduced growth rate. This effect was attributed to the reduced phosphorylation of the CTD of RNAP II (12).

By immunoprecipitation experiments with HA-antibodies using *rct1*^{+/−}pMG1 cell extracts, we show that Rct1 also coprecipitates RNAP II (Fig. 3A). It was therefore of interest to analyze the CTD phosphorylation status in *rct1*^{+/−} cells. Overnight cultures were grown in YES medium to mid-log phase (OD₆₀₀, 0.7), and equal amounts of cells were directly resuspended in SDS-PAGE loading buffer. RNAP II was detected by Western blotting using antibodies specific to the unphosphorylated CTD (8WG16), phosphorylated Ser2 (H5), and phosphorylated Ser5 (H14) (7, 20, 35). Notably, both the H5 and H14 antibodies revealed higher phosphorylation in *rct1*^{+/−} cells, whereas the 8WG16 antibody showed only a modest increase in RNAP II signal (Fig. 3B, lanes 2). This is consistent with the specificities of H5 and H14 antibodies compared to that of 8WG16 (7, 20, 35). Time course analysis of cells grown in YES medium showed that CTD hyperphosphorylation persists from very low OD until the cells reach the stationary

phase (Fig. 3C). Analysis of the cytoplasmic tubulin levels (Fig. 3B and C, lowest panels), as well as staining of membranes with Ponceau S (data not shown), revealed that equal amounts of protein were loaded in each lane. In addition, analysis of the levels of two other RNAP II subunits, Rpb3 and Rpb7, showed that the total levels of RNAP II in WT and *rct1*^{+/−} cells are similar (Fig. 3B). Thus, the results described above clearly demonstrate that the CTD phosphorylation status is impaired in *rct1*^{+/−} cells and that reduced levels of Rct1 result in CTD hyperphosphorylation. Also, these results are consistent with our studies of AtCyp59, whose overexpression led to CTD hypophosphorylation (12).

Episomal expression of Rct1-HA rescues the CTD phosphorylation defect. Next, we analyzed the CTD phosphorylation status in *rct1*^{+/−}pMG1 and *rct1* Δ^{pMG1} strains and compared it to that of WT and *rct1*^{+/−} cells. Cells were grown overnight in YES medium (OD₆₀₀, 0.5 to 0.7 [depending on the strain]), where expression of Rct1-HA from the plasmid is very low, and CTD phosphorylation was analyzed by using H14 and H5 antibodies. Episomal expression of Rct1-HA in *rct1*^{+/−}pMG1 and *rct1* Δ^{pMG1} cells resulted in a decrease in CTD phosphorylation compared to *Rct1*^{+/−} cells (Fig. 3D; compare lanes 2 and 3 with lane 1). However, CTD phosphorylation levels turned out to be even lower than in WT cells. As the same effect was observed in WT cells ectopically expressing Rct1-HA (Fig. 3D; compare lane 5 with lanes 2 and 3), we analyzed Rct1 levels in these strains. As shown in Fig. 3D, Rct1 levels in these strains are somewhat higher than in WT cells (see also Fig. 2A, lower panel on the right). Therefore, these data are consistent with our results with ectopic expression of AtCyp59 in *Arabidopsis* cells, which showed decreased CTD phosphorylation upon a slight increase of protein level (12). Also, this might explain why these cells grow at a lower rate than do WT cells.

To further investigate the effects of Rct1 on CTD phosphorylation, we analyzed CTD phosphorylation in *rct1* Δ^{pMG1} cells in more detail. Cells were grown overnight in YES or EMM medium and then diluted to an OD₆₀₀ of 0.05 in YES containing 100 μ g/ml thiamine, and the same amounts of cells were taken for analysis at the indicated time points. Western blot analysis with anti-HA antibody revealed efficient repression of Rct1-HA expression, resulting in very low levels of Rct1-HA 12 h later (Fig. 3E, lane 4 [HA panel]). Analysis of the same blots with H5 and H14 antibodies revealed a concomitant increase in CTD phosphorylation at Ser2 and Ser5, respectively (Fig. 3E). Again, equal protein loading was controlled by anti-tubulin antibody (Fig. 3E). Thus, the described data clearly show that underexpression of Rct1 increases whereas Rct1 overexpression decreases CTD phosphorylation.

Rct1 is associated with transcriptionally active chromatin throughout the RNAP II transcription cycle. As we were able to demonstrate an interaction of Rct1 with the CTD of RNAP II, we asked next whether Rct1 also associates with active chromatin and at which stage of the transcription cycle. *rct1*^{+/−}pMG1 cells were grown for 16 h under *nmtI*-inducible conditions, and chromatin immunoprecipitation (ChIP) was performed with anti-HA antibodies. Four RNAP II genes were analyzed with oligonucleotides covering promoters, open reading frames, and 3' UTRs. Figure 4A demonstrates that Rct1 is associated with the chromatin at the promoter region and remains associated throughout the transcriptional cycle, as all oligonucleo-

FIG. 3. Phosphorylation status of the RNAP II CTD in *rct1*^{+/-} heterozygous cells. (A) Rct1 coprecipitates RNAP II. Immunoprecipitation of Rct1-HA from *rct1*^{+/pMG1} cell extracts results in coprecipitation of RNAP II, as determined by anti-CTD monoclonal antibody H14. Lane 1, input protein extract; lane 2, protein extract incubated with protein A-Sepharose beads; lane 3, protein extract incubated with anti-HA monoclonal antibody and protein A-Sepharose. Antibodies used for Western blotting are indicated on the right. Immunoprecipitation with anti-HA antibody and protein extract from WT cells did not result in precipitation of detectable amounts of RNAP II (lowest panel, analyzed with anti-CTD H14 antibody). (B) CTD phosphorylation is increased in *rct1*^{+/-} cells. Overnight cultures in the mid-exponential phase (OD₆₀₀ of 0.7) grown in YES medium were collected and directly resuspended in 2× SDS-PAGE loading buffer, and proteins were separated by 8% SDS-PAGE. Blots were probed with monoclonal antibodies against the CTD of RNAP II, Rpb3, and Rpb7 (indicated on the right side of each panel). Analysis of the cytoplasmic α-tubulin level was performed as a loading control. (C) Time course of CTD phosphorylation in WT and *rct1*^{+/-} cells. Overnight cultures grown in YES medium were diluted to an OD₆₀₀ of 0.1 and grown further. Cells were taken at indicated ODs, and CTD phosphorylation was analyzed with monoclonal antibody H14 (specific for Ser5). Analysis of the cytoplasmic tubulin level was used as a loading control (lower panel). Details are as for panel B. (D) CTD phosphorylation defect in *rct1*^{+/-} cells is rescued by episomal expression of Rct1-HA. Protein blots were probed with the antibodies indicated on the right. (E) Repression of Rct1-HA expression increases CTD phosphorylation. Protein blots from *rct1*^{ΔpMG1} cells grown in the presence of thiamine were taken at the indicated time points and analyzed by Western blotting with the antibodies indicated on the right.

tide pairs resulted in a comparable amplification of corresponding DNA fragments in input and coprecipitated DNA samples (compare lanes 3 and 1). Control PCRs performed with oligonucleotides corresponding to promoter and coding regions of the mitochondrial *cox1* gene or the nuclear U6 snRNA gene, which is transcribed by RNAP III, did not result in significant amplification, indicating that Rct1 specifically associates with RNAP II genes. These results are also in agreement with the observation that mainly RNAP II transcription is reduced in *rct1*^{+/-} cells (see below).

To investigate whether association of Rct1 with the chromatin correlates with transcriptional activity, we tested four meiosis-specific genes (*spo4*, *meu13*, *mfr1*, and *mei2* [2, 30]) that showed enhanced transcription when *rct1*^{+/pMG1} cells were grown for 24 h in EMM-N medium (Fig. 2E). We performed ChIP analysis with *rct1*^{+/pMG1} cells grown for 10 and 24 h in EMM-N. Figure 4B clearly demonstrates that 10 h after inoculation, Rct1 is associated to some extent with the promoter, with the coding region, and with the 3' UTRs of the analyzed genes. However, 24 h after inoculation, a significant increase in coprecipitation of promoter, coding regions, and 3' UTRs has been observed (Fig. 4B). In contrast, analysis of the actin gene

(*act1*), which is not affected by meiotic differentiation, did not show any difference between the 10- and 24-h time points (Fig. 4B). Together, these data are consistent with increased transcriptional activity of the *spo4*, *meu13*, *mfr1*, and *mei2* genes at the 24-h time point in *rct1*^{+/pMG1} cells (Fig. 2E) and clearly demonstrate that association of Rct1 with the chromatin depends on its transcriptional activity.

RNAP II transcription is reduced in *rct1*^{+/-} cells. The CTD undergoes a complex pattern of phosphorylation/dephosphorylation during the transcription cycle (see the introduction). In order to enable entry of RNAP II into a new initiation complex, the CTD has to be dephosphorylated after release from the chromatin. Therefore, we investigated the functional consequence(s) of Rct1 depletion on RNAP II transcriptional activity. We measured the levels of nascent transcription for some known RNAP II transcription units by using nuclear run-on analysis. Equal amounts of exponentially growing WT and *rct1*^{+/-} cells were permeabilized with Na-sarcosyl, and ongoing transcription was measured by incorporation of [³²P]UTP. DNA fragments corresponding to the coding regions of 13 randomly selected protein-coding genes were PCR amplified and slot blotted onto membranes. In addition, DNA

FIG. 4. Rct1 is associated with transcriptionally active chromatin throughout the RNAP II transcription cycle. (A) Association of Rct1 with the chromatin was analyzed by ChIP. ChIP was performed with anti-HA antibody and *rct1*^{+/-pMG1} cells grown at 32°C under *nmt1*-inducible conditions (EMM medium). Four RNAP II genes previously analyzed by nuclear run-on were analyzed (upper panels). The two lower panels are controls performed with genes transcribed with mtRNAP (*cox1*) and RNAP III (U6 snRNA). Lanes 1, PCR with immunoprecipitated chromatin; lanes 2, PCR with chromatin bound to protein A-Sepharose alone; lanes 3, PCR with input chromatin. (B) ChIP analysis of four meiosis-specific genes. The *rct1*^{+/-pMG1} cells were grown in EMM-N for 10 or 24 h, and ChIP with the meiotically induced genes *spo4*, *meu13*, *mfr1*, and *mei2* was performed as described for panel A. Coimmunoprecipitated DNA was analyzed by real-time PCR. All samples were run in triplicate to ensure accuracy of the data.

fragments from genes transcribed by RNAP III (tRNA^{Ser} and U6 snRNA), by RNAP I (17S rRNA), and by mitochondrial RNA polymerase (mtRNAP) (*cox1* gene) were employed as controls. Filters were hybridized with total [³²P]UTP-labeled RNA from WT and *rct1*^{+/-} cells and quantified after exposure to a PhosphorImager. Filters were subsequently exposed to X-ray film (Fig. 5A). Quantification of results from three independent experiments clearly showed that transcription of all 13 RNAP II genes is reduced about 20 to 50% in *rct1*^{+/-} cells (Fig. 5A, genes 1 to 13). In contrast, both RNAP III (samples 14 and 16) and mtRNAP (sample 18) transcript synthesis was not affected, whereas RNAP I transcription (sample 15) was also slightly inhibited (Fig. 5A). Thus, transcription run-on data show that partial depletion of Rct1, which results in hyperphosphorylation of the CTD, affects RNAP II transcription. However, an additional effect on RNAP I transcription cannot be excluded (see Discussion).

Because ongoing RNAP II transcription was reduced in *rct1*^{+/-} cells, we wanted to analyze the impact on the steady-state levels of RNAP II transcripts. To do so, total RNA isolated from WT and *rct1*^{+/-} cells was reverse transcribed and used for PCR amplification of 10 RNAP II genes that were previously analyzed by nuclear run-on assay (Fig. 5A, genes 1 to 10). The semiquantitative RT-PCR experiment was repeated several times with newly isolated RNA. Quantification of results shown in Fig. 5B revealed that the RNA steady-state levels for all 10 genes are reduced from 15 to 40% in *rct1*^{+/-} cells. Measurements of ongoing transcription and steady-state levels of mRNAs provide strong evidence that reduction of the Rct1 level results in inhibition of RNAP II transcription. These results, together with the increased phosphorylation status of the CTD, which is predicted to influence its interaction with other proteins, could be the cause of the observed growth and morphological defects of *rct1*^{+/-} cells.

FIG. 5. RNAP II transcription is reduced in *rct1*^{+/-} cells. (A) Autoradiograph of a representative nuclear run-on experiment. Samples 1 to 13, RNAP II transcripts. In addition, DNA fragments from the RNAP I gene (17S rRNA; sample 15), RNAP III genes (tRNA^{Ser} and U6 snRNA genes, samples 14 and 16), the RNAP II gene for U1 snRNA (sample 17), and mtRNAP (*cox1* gene; sample 18), were used as controls. Slot blots were exposed to X-ray film. Quantification of three independent nuclear run-on experiments is shown below. Intensities on the y axis are arbitrary units. (B) Steady-state levels of 10 RNAP II genes (genes 1 to 10 in panel A) were analyzed by semiquantitative RT-PCR. Total RNA isolated from WT and *rct1*^{+/-} cells was reverse transcribed with an oligo(dT) primer, and the resulting cDNA was used for PCR with gene-specific primers. Intensities on the y axis are arbitrary units.

DISCUSSION

The data presented here, together with our previous data on *Arabidopsis* AtCyp59, provide genetic and biochemical evidence that the *S. pombe* nuclear multidomain cyclophilin Rct1 is required for fine tuning of RNAP II CTD phosphorylation. Specifically, we show that *rct1* is an essential gene. Furthermore, deletion of one *rct1* allele in a diploid strain results in elevated phosphorylation of the CTD, reduced RNAP II transcriptional activity, and severe growth and morphological defects. These data, which describe phenotypes of a heterozygote, provide evidence that Rct1 levels are under tight dosage control and that correct levels of Rct1 are absolutely required for a balanced CTD phosphorylation/dephosphorylation cycle. Finally, the presence of Rct1 along the entire length of actively transcribed genes is consistent with a direct interaction between Rct1 and the elongating RNAP II complex. Thus, our results suggest that this multidomain cyclophilin is an essential regulator of RNAP II transcription.

Cyclophilins are ubiquitous proteins that possess PPIase activity; e.g., they catalyze *cis-trans* isomerization of peptide bonds preceding proline. In eukaryotic cells, cyclophilins have been found in all cellular compartments with a variety of functions being ascribed to them, including protein trafficking and maturation, receptor signaling, receptor complex stabilization, apoptosis, RNA processing, spliceosome assembly, and transcription (see references 15, 19, 26, 27, 28, 44, 45, 49, and 52 and references therein). Despite the obviously widespread importance of PPIases, genetic analysis of 13 immunophilin genes in *S. cerevisiae* revealed only *ESS1* (homolog of the human *Pin1*) as essential (13). However, recent reexamination of *ESS1* disruption revealed that it is not absolutely essential for cell viability and that its deletion rather causes a severe temperature-sensitive growth phenotype (18). *ESS1* is also not essential in *S. pombe* (18) and *Cryptococcus neoformans*, but it is in some other fungi, e.g., *Candida albicans* and *Aspergillus nidulans*. Similarly, Rct1 is essential in *S. pombe*, but its homolog in *P. tetraurelia*, Kin241, is not (22). It is not clear why deletion of a particular PPIase is lethal in some organisms and not in others. One possibility is that in certain genetic backgrounds, high expression levels of other PPIases compensate for the loss of the other(s), thus acting as natural suppressors. This would also indicate that the functions of certain PPIases are partially redundant or overlapping. Alternatively, it is possible that some PPIases are essential only under certain conditions.

The highly repetitive primary sequence of the CTD suggests that it can adopt different conformations. The significance of Rct1 regulating CTD function is very convincing in light of recent structural data that suggest that the free CTD, which contains a multitude of heptapeptide repeats (YSPTSPS), is highly flexible. Significantly, the peptidyl-proline isomerase Pin1, the capping enzyme guanylyltransferase Cgt1, and Pcf11, an essential component of the mRNA cleavage factor IA, all bind to CTD peptides only in an all-*trans* conformation in vitro, providing the first direct evidence for the importance of *cis-trans* isomerization of prolyl bonds in CTD repeats in protein-protein interactions (10, 31, 32, 34, 48). Furthermore, interactions of the CTD with regulatory factors have been found to depend on Ser2 and Ser5 phosphorylation (23, 37, 38). In

FIG. 6. Model of how Rct1 might influence CTD structure and function. We propose that the “CTD code” is a result of coordinated activities of CTD kinases, phosphatases, and PPIases, which together generate high-affinity binding sites for factors involved in the regulation of RNAP II transcription and its coupling with pre-mRNA processing.

particular, it has been shown that Pcf11 binds the CTD in a phosphorylation-dependent manner, whereby phosphorylation of the two adjacent repeats contributes to an increase in the affinity of Pcf11 for CTD (34). These new crystallographic and biochemical data provide evidence of how changes in phosphorylation and proline isomerization could contribute to the structural plasticity of the CTD, which is important for recruitment of many different proteins to the CTD and the transcription complex (3, 24, 32). These prerequisites suggest that kinases, phosphatases, and peptidyl-prolyl isomerases are important regulators of CTD functions (3, 24, 32, 34).

Several kinases and phosphatases have been shown to influence phosphorylation/dephosphorylation of the CTD (32, 34, 38), but only one immunophilin, Pin1/ESS1, has been implicated in the regulation of these processes (50, 51). Pin1 is a small parvulin-type immunophilin that has been shown to interact with many different proteins involved in a wide variety of cellular processes (4, 27, 28, 52). As *pin1*^{-/-} cells accumulate hypophosphorylated RNAP II, whereas moderate overexpression of Pin1 resulted in the appearance of a novel hyperphosphorylated form of RNAP II (51), its influence on CTD phosphorylation was investigated. In vitro experiments suggested that human Pin1 inhibits the CTD phosphatase FCP1 and stimulates CTD phosphorylation by cdc2/cyclin B (51). Here, we show that reduced levels of the cyclophilin Rct1 result in increased CTD phosphorylation at both Ser2 and Ser5, whereas increased levels of Rct1 (and also of AtCyp59) (12) reduced CTD phosphorylation. This indicates that, under physiological conditions, Rct1 stimulates CTD dephosphorylation. Thus, these two PPIases have entirely opposite effects on the CTD phosphorylation status; Rct1p has a positive effect on CTD dephosphorylation (this work), as opposed to Pin1, which has a negative effect (51). However, there is genetic evidence from *S. cerevisiae* indicating that ESS1 stimulates dephosphorylation of the CTD (50), as Rct1 does in *S. pombe*. Nonetheless, in both cases it is not clear how these effects are brought about. Rct1 could either bind to the CTD, thereby facilitating access of other CTD-modifying enzymes, or, alternatively, the CTD kinases and phosphatases are direct targets of Rct1 (Fig. 6). Intriguingly, PTPA, an unusual PPIase, has recently been shown to function as an activator of PP2A phosphatase (21).

The increase/decrease in CTD phosphorylation by reducing/elevating the level of Rct1 indicates that Rct1 could be involved in either inhibiting a CTD phosphatase or stimulating a CTD kinase activity. However, further experiments are necessary to answer these questions more precisely.

As *cis-trans* isomerization of peptide bonds takes place spontaneously, but at a much lower rate (42, 44, 49), it is also possible that PPIases are not absolutely required *in vivo*. Rather, they might be generally required for selective prolyl peptide bond isomerization in processes where fast changes in protein-protein interactions or protein phosphorylation/dephosphorylation take place, e.g., coupled transcription and pre-mRNA processing. Many genetic and biochemical data have accumulated over the last decade that indicate the physical and functional coupling of RNAP II transcription with pre-mRNA processing and chromatin structure through the CTD of RNAP II. A highly dynamic CTD phosphorylation pattern is required for coordination of transcription and nuclear pre-mRNA processing (1, 4, 17, 35, 37, 39, 46, 53). In addition, it has recently been demonstrated that *cis-trans* isomerization of prolyl peptide bonds may also contribute to establishing or disrupting binding interactions with the CTD (3, 24, 32, 34). This, together with the data presented here and previously with Pin1, highlights the general importance of PPIases in the regulation of CTD structure and function (Fig. 6).

We were able to show here that Rct1 is required for correct CTD phosphorylation and consequently for efficient RNAP II transcription. However, the highly pleiotropic phenotype observed with *rct1*^{+/-} cells, together with the observed tight dosage control of Rct1, is unusual for PPIases and hints at another, as-yet-unknown function(s) besides RNAP II transcription regulation. Indeed, we show that RNAP I transcription is reduced as well in *rct1*^{+/-} cells. The reason for the decrease in transcription of the 17S rRNA is not clear at the moment. However, this is consistent with our previous observation that green fluorescent protein-tagged AtCyp59 localizes to the nucleolus to some extent (12), where it could possibly affect transcription of RNAP I as well. Also of note is that deletion of one *rct1* allele, which results in reduced Rct1 protein levels, causes strong growth and morphological phenotypes, presumably due to deregulated CTD phosphorylation and RNAP II transcription. However, the possibility that Rct1 is also involved in regulation of factors important for cell morphology and differentiation is not excluded. Episomal expression of Rct1-HA in *rct1*^{+/-pMG1} cells partially restored all growth and morphological defects observed in *rct1*^{+/-} cells, whereas moderate overexpression in WT cells caused a strong negative effect on their growth and meiotic differentiation, although cell size, polarity, and mitotic division were as in WT cells. Thus, Rct1 levels are clearly under tight dosage control.

Rct1 is an unusual cyclophilin, as it contains an RRM and a C-terminal domain rich in RS/RD dipeptide repeats and charged amino acids. The high conservation of these multidomain proteins, in particular in the RRM region, suggests additional possible functions apart from PPIase activity. Indeed, our observation that AtCyp59 also interacts with SR proteins in *Arabidopsis* suggested that it might regulate events at the interface of transcription and splicing (12). In fact, the observed meiotic phenotype in *rct1*^{+/-} cells might be explained by the deregulation of CTD phosphorylation and concomitant

changes in binding of proteins regulating splicing of meiosis-specific genes. Some meiosis-specific genes are transcribed under vegetative growth; however, their pre-mRNAs are spliced only upon meiotic induction (2, 30). The factors regulating these events may bind the CTD only under specific conditions. Thus, the deregulation of CTD phosphorylation observed in *rct1*^{+/-} cells might induce their binding under vegetative growth, consequently leading to splicing and induction of meiosis. Similarly, it has recently been demonstrated that the CTD promotes alternative splicing of fibronectin pre-mRNA by recruiting SR protein SRp20 (9). Interestingly, the human homolog of Rct1, PRIL4, has recently been identified in affinity-purified spliceosomal B complex (8), further supporting our hypothesis that Rct1 plays an important role at the interface of transcription and pre-mRNA processing. Whether Rct1 is indeed involved in pre-mRNA processing and, if so, how it contributes to splicing regulation requires further investigation.

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